STUDIES OF GLUTEN SENSITIVITY

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SUMMARY

Coeliac disease is defined as a permanent gluten sensitive enteropathy. The classical mucosal lesion is of villous atrophy, crypt hyperplasia and lymphocyte infiltration of the epithelium. It is now generally agreed that a spectrum of mucosal damage exists ranging from total villous atrophy to normal villous architecture with increased numbers of intraepithelial lymphocytes (IEL). Gluten sensitivity can still be demonstrated in this minimal lesion, with the IEL count falling on withdrawal of gluten and rising again on gluten challenge. In the first part of this thesis I present work validating computerised image analysis as a method for quantifying IEL in small bowel biopsy in order to identify individuals with minimal change gluten sensitive pathology.

Antibodies to the gliadin fraction of gluten and also to an unidentified tissue antigen occur in coeliac disease. These antibodies are useful in the initial diagnosis of the disease as well as in monitoring response to treatment and timing of biopsy following oral gluten challenge. They are also used in the screening of individuals who have a higher incidence of coeliac disease. In Chapter 4 I describe the setting up of a recently developed assay for IgA endomysium antibody using human umbilical cord, both for research and also for the routine NHS clinical service in Lothian. I have also adapted this method for use in IgA deficient individuals by measuring the IgG isotype of this antibody.

In other centres IgA endomysium has been used to identify previously undiagnosed cases of coeliac disease in populations at high risk of developing the disease, such as family members of coeliacs or individuals with insulin-dependent diabetes. In Chapter 5 I describe our use of endomysium and antigliadin antibodies as screening tools in a general hospital population, individuals with gastrointestinal disease and those with type I diabetes. Through the course of this work I found that a high number of patients with chronic liver disease had positive IgA endomysium antibody assays with raised titres of IgA antigliadin antibodies without evidence of coeliac disease on small bowel biopsy. By investigating this further I found these individuals demonstrated an atypical pattern of fluorescence within the smooth muscle of the umbilical vessels.

Progress in the application of strict criteria for the diagnosis of gluten sensitivity has been hampered by the lack of a rapid and reliable diagnostic test. Currently the only evidence of gluten sensitivity is based on clinical and pathological effects of gluten withdrawal and challenge, and this may take several months or years to develop, requiring multiple invasive small bowel biopsies. For this reason rectal challenge with gluten is being considered as a possible alternative method for demonstrating gluten sensitivity. Currently this requires computerised image analysis of multiple rectal biopsies. In the final part of this thesis I describe an attempt at performing rectal gluten challenge using a wash technique with a polyethylene glycol and electrolyte solution. This wash was performed before and after instillation of gluten into the rectum and the fluid retrieved was analysed for inflammatory proteins, immunoglobulins and cytokines.

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ABBREVIATIONS

α-1-AT	Alpha-1-antitrypsin
Alb	Albumin
AGA	Antigliadin antibody
APC	Antigen presenting cell
BSA	Bovine serum albumin
CIA	Coeliac like intestinal antibody pattern
ELISA	Enzymed linked immunosorbant assay
DH	Dermatitis Herpetiformis
ESPGAN Gastroenterology	European Society of Paediatric and Nutrition
FITC	Fluoresceinisothiocyanate
GALT	Gut associated lymphoid tissue
GFD	Gluten free diet
GI	Gastrointestinal
H+E	Haematoxylin and eosin
Hb	Haemoglobin
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocyte
Ig	Immunoglobulin
IL	Interleukin
MHC	Major histocompatibility complex
ND	Normal diet
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PEG+E	Polyethylene glycol plus electrolytes
PVA	Partial villus atrophy
STVA	Sub-total villus atrophy
TVA	Total villus atrophy
WGLF	Whole gut lavage fluid

PRESENTATIONS

The following presentations have been based on the work contained within this thesis:

1. **Quantification of Intraepithelial Lymphocytes using Computerised Image Analysis**. Poster presentation at the *Seventh International Symposium on Coeliac Disease, Tampere, Finland. September 1996*

2. **Chronic Liver Disease may be a cause of False Positive Results in the Antiendomysium Antibody Assay**. Poster presentatio at the *Seventh International Symposium on Coeliac Disease, Tampere, Finland. September 1996*

3. **Screening for Coeliac Disease in the Paediatric Insulin-Dependent Diabetic Population of South-East Scotland using Fingerprick Blood Samples**. To be presented at the meeting of the *European Society for Paediatric Endocrinology, Stockholm, June 1997.*

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DECLARATION

I declare that the work contained within this thesis is all my own, with any exceptions clearly marked.

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FOREWORD AND AIMS

Coeliac disease is defined as a permanent gluten sensitive enteropathy. It is now generally agreed that the enteropathy may be expressed only as a high count of intraepithelial lymphocyte (IEL) which rise with gluten loading and fall on a gluten free diet. Separate studies have shown an unusual serum antibody in untreated coeliac patients, endomysium antibody (EmA) and there are people who have positive EmA and virtually normal biopsies. Some of these at least have however been found to have a high count of IEL. These and other studies such as in immunogenetics are suggesting that we may have to widen our definition of coeliac disease and gluten sensitivity, and be more aware of the possibility of a role of gluten in patients who do not have classical pathology. Progress in the application of strict criteria of gluten sensitivity has been hampered by the fact that currently the only evidence is based on clinical and pathological effects of gluten withdrawal and challenge which may take months or years and require multiple invasive small bowel biopsies. For this reason rectal challenge with gluten is being considered as a possible alternative method for demonstrating gluten sensitivity. Currently, this requires computerised image analysis of multiple rectal biopsies and is clearly not widely applicable.

Against this background my research has addressed a number of aspects relevant to the subtle expression of gluten sensitivity, both in terms of positive serological tests and minor pathological changes, and also subtle clinical expression which has previously been unrecognised and can be detected in screening programmes.

My specific aims were:

1. To develop a scientifically valid method for characterising the most mild of pathological abnormality within the spectrum of expression of coeliac disease. I therefore set out to use computerised image analysis to obtain counts of IEL in small bowel biopsies.

2. An inexpensive method for detecting endomysium antibody was described shortly after I took up my research post and I set out to develop this assay for use in my research and also for NHS clinical service use in Edinburgh. My aim was to further refine serological tests so that they could be applied in screening, for example, in patients attending for GI tests or in groups known to have a higher than normal incidence of coeliac disease.

3. Colleagues in the GI Laboratory already had considerable experience in the use of a polyethylene glycol based gut lavage fluid for measuring intestinal inflammation and immunity and I set out to establish whether a simple rectal wash technique could be used to detect gluten sensitivity and ultimately to investigate forms of tissue damage and immunoregulatory and inflammatory molecules involved.

CHAPTER 1: GENERAL INTRODUCTION

Definition and Pathology

Coeliac disease, or gluten sensitive enteropathy, can be defined as a chronic disease in which there is a characteristic, though not specific, mucosal lesion of the small intestine that impairs nutrient absorption by the involved bowel (picture 1.1). There is prompt improvement of both the absorption and the mucosal lesion on withdrawal of wheat gliadins and the equivalent barley, rye and oat prolamins from the diet (Trier, 1991) with relapse occurring on their dietary reintroduction.

Gluten sensitivity is associated with a spectrum of mucosal lesions, and it would appear that all these lesions comprise a dynamically interrelated series of events (Marsh and Crowe, 1995). Classically, in untreated coeliac disease the small intestinal mucosa is flat with flattened, or cuboidal, enterocytes and hyperplastic crypts (Marsh, 1972). There is an infiltration of intraepithelial lymphocytes (IEL), which display an increased rate of mitotic activity (Marsh, 1982).

Fry et al (1972; 1974) described marked infiltrations of intraepithelial lymphocytes within a structurally normal mucosa in proximal small bowel biopsies from patients with dermatitis herpetiformis (DH). These lesions improved on withdrawal of gluten from the diet and gluten-associated IEL infiltration with otherwise normal pathological and histological appearances is now recognised in individuals without DH. This infiltration is also seen in the presence of crypt hyperplasia and graft-versus-host reactions in experimental animals suggest this is a local T-cell-dependent response of the intestinal mucosa to foreign antigen (Mowat and Ferguson, 1982). IEL infiltration probably does not damage the mucosa as there is no evidence of structural or functional impairment. In addition to gluten sensitivity, infiltration may occur in tropical sprue, parasitic or viral infection, AIDS enteropathy or graft-versus-host disease (Marsh and Crowe, 1995). There have been recent reports of high numbers of IEL occurring in the presence of the coeliac associated endomysium antibody (Picarelli et al, 1996), and this may prove

Picture 1.1 Classical histology of coeliac disease showing subtotal villous atrophy with crypt hyperplasia and increased numbers of intraepithelial lymphocytes

to be a useful tool in the diagnosis of low grade gluten sensitive enteropathy.

Genetics of Coeliac Disease

T-cells are unable to recognise antigen directly but require it to be presented bound to a cell-surface human leukocyte antigen (HLA) molecule on an antigen presenting cell (APC). The HLA molecules can be divided into two classes, reflecting differences in tissue expression, antigen processing and presenting pathways, and in T-cell receptor (TCR) recognition. Class I molecules possess a binding site for the T-cell accessory molecule CD8, and so tend to present antigen only to $CD8^+$ T-cells. Class II molecules do not interact with CD8, but do so with CD4 (Tighe and Ciclitira, 1995). The genes encoding for components of the antigen-presenting system are located mainly on the short arm of chromosome 6 in the region termed the major histocompatibility complex (MHC). The class I region spans 1.5 million base pairs and includes the classical class I loci, HLA-B, -C, and -A in addition to non-classical loci HLA-E to HLA-H and genes of unknown function. The class II region includes the three classical HLA class II loci, HLA-DR,-DQ and -DP as well as genes involved in antigen processing for class I presentation. Separating these two regions is the class III region which includes genes whose products have immunological functions eg tumour necrosis factors, heat-shock proteins along with other loci whose functions are as yet unknown (Tighe and Ciclitira, 1995).

In 1972 an association was found between the class I allele HLA-B8 and coeliac disease (Stokes et al, 1972). Subsequent studies have shown this association to be with the HLA-B8, DR3 haplotype, with a stronger relative risk ascribed to the DR3 allele than HLA-B8 (Keuning et al, 1976). In Southern Europe, where the prevalence of the HLA-B8, -DR3 haplotype is lower than in the UK there is an association between coeliac disease and the haplotype HLA-B18, -DR3, -DQ2, indicating that the HLA predisposition resides within the common class II region rather than the differing class I or III regions (Rittner et al, 1984; Coniga et al, 1992).

Other populations within Southern Europe exhibit an association between coeliac disease and HLA-DR7 (Albert et al.1978; DeMarchi et al, 1983). This association only occurs in the presence of HLA-DR5 or -DR3, and similarly no association is found with DR-5 in the absence of -DR7. DR3 haplotypes all possess the DQ alleles DQA1*0501 DQB1*0201. DR5 haplotypes possess the DQ allele DQA1*0501 but the DQB1*0301 allele in place of DQB1*0201 whereas DR7 haplotypes lack the DQA1*0501 but do have the DQB1*0201. A heterozygous combination of the DR5/DR7 haplotypes, therefore contains the same DQA1*0501 DQB1*0201 alleles as the DR3 haplotype. This allele combination is found in 98% of coeliacs in northern Europe and in 92% of those from southern Europe and has been proposed that these DQ alleles are the primary HLA susceptibility genes of coeliac disease (Tighe et al, 1992; Sollid et al, 1989). It is therefore proposed that the definition of coeliac disease be expanded to include the presence of DQ2, and hopefully in the next few years this will be widely accepted.

The work presented in this thesis studied three aspects of gluten sensitivity - the small intestinal intraepithelial lymphocyte, endomysium antibodies and rectal gluten challenging. It is hoped that the last of these will be of use in the diagnosis of the minor pathology variants of coeliac disease.

Small Bowel Intraepithelial Lymphocytes

Intraepithelial lymphocytes (IEL) are interspersed between and in close contact with the epithelial cells (picture 1.2). The nuclei are densely stained and cytoplasm pale and featureless on light microscopy (Ferguson, 1977). Many of the lymphocytes can be seen to cross the basement membrane between the epithelium and lamina propria, probably in both directions. IEL are primarily situated in the basal regions of the epithelium suggesting they are not migrating into the gut lumen from the lamina propria. In coeliac disease markedly increased numbers of small intestinal IEL are seen and although these fall with treatment, they rarely reach normal levels (Ferguson and Murray, 1971). Raised IEL counts of similar magnitude have also been reported

in tropical sprue and giardiasis (Montgomery and Shearer, 1974; Ferguson et al, 1976).

Human IEL are predominantly (over 95%) $CD3^+CD2^+$ T cells (Trejdosiewicz and Howdle, 1995). 70-90% of IEL T cells are $CD8^+$, suggestive of a cytotoxic/suppressor function, the majority of the rest are $CD4^+$ (helper) cells, with a small minority (less than 5%) of "double negative" or $CD4^-CD8^-$ cells. The $CD8^+$ subset tend to express CD5 at reduced density compared to peripheral lymphocytes. CD5 is important for T-B cell interactions, suggesting that IEL T cells have little involvement with B cells. The majority of IEL T cells are $CD45RO^+CD45RA^-$, a phenotype reflecting prior contact with antigens (Cerf-Bensussan et al, 1993).

The majority of small intestinal IEL express the $\alpha\beta$ form of T cell receptor, with only 1-10% expressing the $\gamma\delta$ form (Trejdosiewicz and Howdle, 1995). In coeliac disease there is an increase in the abundance and percentage of $\gamma\delta^+$ IEL and 90% of these cells are $CD8^-$ (Halstensen et al, 1989). This increase persists irrespective of dietary gluten and is seen even in well treated patients with normal or near-normal morphology. The tendency to high counts of $\gamma\delta^+$ IEL has also been described in latent coeliac disease (Mäki et al, 1991b), in dermatitis herpetiformis (Savilahti et al, 1992) and in those thought to be genetically predisposed to coeliac disease (Holm et al, 1992). The significance of the increase in $\gamma\delta^+$ T cells is, as yet unclear, but they may have an "immune surveillance" role.

The functions of IEL are not yet fully understood. Human IEL demonstrate no spontaneous cytotoxicity towards the natural killer target K562 and only weak cytotoxicity towards colonic tumour cell lines, but their cytotoxicity can be boosted by incubation with phytohemaglutinin, anti-CD2 or anti-CD3 (Taunk et al, 1992; Ruthlein et al, 1992). Cytotoxic IEL express CD2, CD3 and CD8 whereas peripheral cytotoxic lymphocytes express CD2, CD16 and NKH1. In vivo, isolated $\alpha\beta^+$ IEL exert a strong cytotoxicity against targets coated with enterotoxins suggesting that they can rapidly develop TCR mediated cytotoxicity (Cerf-Bensussan et al, 1993). IEL, therefore, may

Picture 1.2: Normal small bowel histology with enlargement demonstrating the appearance of IEL

well play a role in the elimination of damaged or infected enterocytes. Unseparated human IEL synthesize IFN-γ and TNF-α (Ebert, 1990). Cytokine production may play a role in protecting the gut from bacterial and viral infection, but exaggerated secretion of IFN-γ may increase epithelial cell permeability deleteriously (Hiribarren et al, 1993). IEL may also have a role in the development of oral tolerance to dietary antigens; injection of murine $\gamma\delta^+$ cells abrogated oral tolerance to a soluble antigen (Fujihashi et al, 1992).

Coeliac Disease Antibodies

The majority of foreign antigens are encountered at mucosal surfaces, and 80% of all immunoglobulin-producing cells in humans are located in the intestinal mucosa. In order for antibody production to occur the antigen must first be phagocytosed and processed by antigen presenting cells (APC) such as B cells, macrophages, dendritic cells and the intestinal epithelial cell. In addition, in the gastrointestinal tract, M cells, which overlie the Peyer's patch, are thought to sample antigens in the gut lumen and pass them unchanged into the lamina propria and the APCs (Mayer, 1991). The antigenic peptides are then presented by the human leukocyte antigen (HLA) type II molecules, products of the major histocompatibility complex (MHC), situated on the cell surfaces. These molecules contain a peptide-binding groove where the T cell epitope (the processed peptide of 10-20 amino acids) is situated for recognition. The HLA class II molecule and peptide complex interact with $\alpha\beta^+CD4^+$ (helper) cells. This T cell activation signals B cells to transform into antibody producing cells (Parker, 1993; Clark and Ledbetter, 1994).

Intestinal immunity revolves around the maturation cycle of specifically primed T and B cells from the gut associated lymphoid tissue (GALT) via the mesenteric lymphatics and the peripheral blood back to the intestinal lamina propria ("homing"). The Peyer's patches switch the B cells to the IgA isotype during the maturation process (Mäki, 1995).

Serological tests, at present, do not replace the need for small bowel biopsy in the diagnosis of coeliac disease, especially in those individuals with a strong clinical suspicion of the disease, but they do play a major role in the monitoring of treatment and also in screening individuals in whom there is only a slight clinical suspicion of coeliac disease. More and more these tests are being used to screen groups of individuals at higher risk of the disease.

Isotype-specific antigliadin antibody tests are the most widely used at present (Troncone and Ferguson, 1991). These antibodies have frequently been found in patients with untreated coeliac disease, but the sensitivity and specificities of the assays vary from 30-100% (Ascher et al, 1990; Bodé and Gudmand-Hoyer, 1994; Ferreira et al, 1992; Friis and Gudmand-Hoyer, 1986; Kilander et al, 1987; McMillan et al, 1991; Not et al, 1993; Ståhlberg et al, 1986; Mäki et al, 1991c). Antigliadin antibodies may be found in other conditions as well as coeliac disease and dermatitis herpetiformis, such as inflammatory bowel disease, cows' milk enteropathy, atopic eczema, pemphigoid and rheumatoid arthritis (Burgin-Wolff et al, 1983; Unsworth et al, 1983; Kieffer and barnetson, 1983; Finn et al, 1985; O'Farrelly et al, 1988) and also in healthy individuals (Grodzinsky et al, 1990; Uibo et al, 1993; Pettersson et al, 1993). Titres of antigliadin antibodies decline with treatment with a gluten free diet (Savilahti et al, 1983; Kilander et al, 1987) and rise again with the reintroduction of gluten in the diet, and may predate mucosal or clinical relapse by several months (Scott et al.1992). High titres of IgG antigliadin antibodies persist for longer after treatment than IgA class antibodies (Troncone and Ferguson, 1991; Kilander et al, 1987). The development of antigliadin antibodies is independent of HLA type (Pettersson et al, 1993).

Tissue antibodies in coeliac disease were first described in 1971 in the form of the reticulin antibody test (Seah et al, 1971). Antibodies were detected using a standard indirect immunofluorescence method using sections of rodent kidney, liver and stomach as antigenic substrate. A particular staining pattern was recognised in coeliac disease and dermatitis herpetiformis (Rizzetto and Donach, 1974) which improved the sensitivity and specificity of the assay. It was noted that in untreated

coeliac disease reticulin antibodies were primarily of IgA class (Magalhaes et al, 1974), and a sensitivity of 97% and specificity of 98% were claimed (Mäki et al, 1984b). IgA reticulin antibodies have been found to be reliable in the detection of occult coeliac disease (Hällstrom, 1989; Collin et al, 1990; Unsworth and Brown, 1994) and have been used to screen populations at increased risk of coeliac disease, for example, insulin-dependent diabetics (Mäki et al, 1984a; Collin et al, 1989), Sjögren's syndrome (Collin et al, 1992a) and autoimmune thyroid disease (Collin et al, 1994). In selective IgA deficiency, IgG reticulin antibodies have been found to be predictive for coeliac disease (Collin et al, 1992b).

Serum from coeliacs also reacts with human tissue. Hällstrom (1989) showed that all reticulin antibody-positive sera produced a strong immunofluorescent pattern with human jejunum, liver, lung and spleen. Reticulin antibodies can, therefore be tested for using human jejunum as the substrate, and in this case the test is called the jejunal antibody test (Kárpáti et al, 1990). Chorzelski et al (1983; 1984) used monkey oesophagus to test serum from patients with coeliac disease and dermatitis herpetiformis and named it the endomysium antibody test. Endomysium antibody is directed against "reticulin-like" silver-stain positive fibres in the connective tissue of the oesophageal smooth muscle called endomysium (Kumar et al, 1987). This IgA-class tissue antibody has gained popularity as it gives almost 100% sensitivity and specificity in coeliac disease (Ferreira et al, 1992; Hällstrom, 1989; McMillan et al, 1991; Mäki et al, 1991c; Kapuscinska et al, 1987), but its use has been limited due to the high cost of commercial kits. More recently, however a technique has been described to detect endomysium antibodies using indirect immunofluorescence with fixed cryostat sections of human umbilical cord as the antigenic substrate without a fall in its accuracy (Ladinser et al, 1994). This has dramatically reduced the cost of the test and enabled many more centres to use testing of endomysium antibodies as a first line investigation in individuals with a clinical suspicion of the disease as well as individuals at high risk of developing the disease.

In dermatitis herpetiformis, two thirds of patients will show evidence of coeliac disease on small bowel biopsy (Fry et al, 1974), but the proportion of individuals with abnormal

biopsies increases towards 100% if multiple specimens are taken suggesting that the lesion is more patchy than in coeliac patients (Brow et al, 1971). If endomysium antibody is looked for in all patients with DH on a normal diet, 65% will be positive for the antibody. Only 86% of those with STVA have IgA class endomysium antibody and only 11% of those with PVA (Volta et al, 1992). Chorzelski et al (1983) found that 68% of all DH patients had IgA EmA but a higher incidence of 79% was found by the same group in a later study (1984).

Tissue antibodies in coeliac disease and DH are gluten induced, ie they are found in untreated patients and disappear within a year of treatment with a gluten free diet (Mäki et al, 1984b; Hällstrom, 1989). They reappear on reintroduction of a normal diet or on the relaxation of gluten free diet and have therefore been used to predict mucosal relapse on oral gluten challenging (Kapuscinska et al, 1987; Mäki et al, 1989).

Reticulin and endomysium antibodies are directed against human "reticulin" and have been interpreted as being the autoantibodies of coeliac disease (Kárpáti et al, 1990). These antibodies have been shown to be generated against a synthetic product of human fibroblasts (Marttinen and Mäki, 1993). These molecules absorbed the IgA antibodies responsible for reticulin and endomysium antibody positivity but not the IgA responsible for gliadin antibody positivity.

In untreated coeliac disease there are increased numbers of IgA-, IgM- and IgG-producing plasma cells in the jejunum. The intestinal fluid contains high levels of antigliadin antibodies (Volta et al, 1988) but, unlike serum antigliadin antibodies, the intestinal antibodies do not fall to normal levels on a gluten free diet (O'Mahony et al, 1991). IgA-class reticulin antibodies have also been described in jejunal fluid (Mawhinney and Love, 1975) suggesting an intestinal origin for these antibodies. In serum, however the reticulin or endomysium antibodies are predominantly the IgA_1 subclass with a ratio of IgA_1:IgA_2 antibodies similar to the proportion of total serum IgA_1:IgA_2 suggesting a systemic origin (Garrote et al, 1991).

Local humoral responses can be studied by measuring antibodies in jejunal aspirate taken at the time of jejunal biopsy by capsule or in whole gut lavage fluid (WGLF) (O'Mahony et al, 1990; O'Mahony et al, 1991). These studies showed that IgA in WGLF is predominantly secretory type. Elevated levels of IgA and IgM were observed in untreated coeliac disease, and intestinal fluids contained IgA and IgM antigliadin antibodies as well as antibodies to β-lactoglobulin and ovalbumin. Secretory IgM antibody activity persisted despite treatment with gluten free diet and was unrelated to the degree of enteropathy. This polyclonal upregulation of mucosal IgM responses was defined as the "coeliac-like intestinal antibody" pattern (CIA) and may be one of the methods used to identify individuals who may be gluten sensitive but have normal small intestinal biopsies (potential coeliac disease).

Tissue antibodies do occasionally occur in individuals with normal small intestinal mucosal architecture and at present the diagnosis in these individuals is uncertain. They may, however represent individuals with latent coeliac disease, in whom the mucosa at a later date will deteriorate and develop villous atrophy (Ferguson et al, 1993; Mäki et al, 1990). Persistently raised IgA antigliadin antibodies predict the later development of coeliac disease in only 24% of patients, and IgG antigliadin antibodies not at all. Positive reticulin antibodies, however, predict later development of coeliac disease in 83% of cases (Collin et al, 1993) whereas the majority of patients with or without abdominal symptoms but persistently elevated IgA antigliadin antibody titres probably do not have latent coeliac disease (Uibo et al, 1993). It may be argued that individuals should be treated before the development of symptoms or malabsorption (Arranz and Ferguson, 1993), and tissue antibody tests may provide a mechanism for identification of these patients.

Although gliadin antibodies occur in other disorders apart from coeliac disease and dermatitis herpetiformis and they are not limited to individuals with gene markers of coeliac disease susceptibility (Mäki et al, 1991c; Pettersson et al, 1993) they may still play a role in the pathogenesis of coeliac disease. Gluten induces IgG-mediated subepithelial complement activation and deposition of terminal complement

complexes, and the deposition correlates with the number of mucosal IgG cells, the serum levels of gluten-specific IgG and IgM antibodies and the degree of villous atrophy (Halstensen et al, 1992). Gluten, therefore, may damage the surface epithelium in coeliac disease via immunoglobulin-mediated subepithelial complement activation. Gliadin antibody could also cause mucosal damage via cell-mediated cytotoxicity in which the antibody recognises gliadin peptides bound to the mucosal structures (Unsworth et al, 1987) and directs an antibody-dependent cell-mediated cytotoxic reaction.

Reticulin or endomysium antibodies may represent an autoantibody responsible for initiating and perpetuating the intestinal damage seen in coeliac disease. Family studies have shown the HLA class II molecule DQ to be the restriction element for these antibodies (Mäki et al, 1991a). Mäki et al (1991a) have based their hypothesis for coeliac disease being an autoimmune disease on the identification of self-peptides which trigger the production of reticulin and endomysium antibodies. This hypothesis depends on three crucial elements; the trigger (gliadin), the susceptibility MHC class II genes (DQA/DQB) and the autoantigen. Specific HLA class II molecules on APC may present self-peptides, thus activating the autoreactive T-cell population. The disease is self-perpetuating if the trigger is not removed. The exact nature of the self-peptides are yet to be determined, but may be fibroblast-derived extracellular matrix proteins (Marttinen and Mäki, 1993). A reported case of coeliac disease and hypogammaglobulinaemia (Webster et al, 1981), however, suggests that humoral immunity is not crucial for the development of the mucosal lesion.

Although the humoral immune system has been implicated in the development of coeliac disease (Brandtzaeg et al.1993), it may be secondary to T-cell activation and helper-cell recruitment. Humoral responses may be important in amplifying the immune response and proliferation of the mucosal damage. The development of tissue antibodies may represent the consequence of T-cell mediated damage, exposing the self-peptides and allowing the development of autoantibodies (Marsh et al, 1993). The lower prevalence of IgA endomysium antibodies in patients with DH may be due to the more limited extent of bowel damage occurring in DH and this,

therefore supports the theory that endomysium antibodies are a consequence of cell mediated bowel damage rather than being responsible for initiating the mucosal lesion.

Rectal Gluten Challenging

Direct instillation of gluten into the ileal lumen in coeliacs induces rapid deterioration in the mucosal architecture and it has been postulated that the rapid breakdown of gluten in the proximal small intestine produces relative sparing of the distal small intestine and that a similar mechanism may protect the colon (Flick et al, 1962). The same group found abnormalities in rectal biopsies from 5% of coeliac patients namely, increased tortuosity of the glands with branching and crypt abscesses and frequent polymorphonuclear lymphocytes within the lamina propria (1962). The surface epithelium was disorganised in appearance and in some patients was partially denuded. Ensari et al (1993) found significant increases in $CD3^+$ and $\gamma\delta^+$ lymphocytes within the lamina propria and epithelium of untreated patients compared with treated coeliacs but neutrophils ($CD15^+$) were not prominent. Activated ($CD25^+$) lymphocytes expressing IL-2 receptors were increased in the lamina propria, but few IL-$2R^+$ IEL were found. The absence of neutrophils suggests this is not a conventional inflammatory proctitis, but a cell mediated response to gluten antigens present in the faecal stream.

A small group of coeliac patients given wheat enemas with rectal biopsies before and at 4, 8 and 24 hours developed histological, sigmoidoscopic and clinical reactions (Dobbins and Rubin, 1964). Erythema, maximal at 8 hours was seen on sigmoidoscopy. Histologically, polymorphonuclear infiltration of the epithelium, lamina propria and submucosa occurred and surface goblet cells were discharged. These changes were maximal at 8 hours and resolving at 24 hours. Two of the four coeliac patients developed clinical responses to the wheat enemas consisting of anorexia, nausea, vomiting, borborygmi, loose stools and headache. Corn enemas produced no reaction in the coeliac group, and the control group did not respond to either enema. The biopsies taken in this study were later reviewed (Austin and Dobbins, 1988) and

IEL counted. The IEL count of the baseline biopsies was significantly higher in the coeliac group than in the controls and the wheat enemas induced a significant increase in IEL in the coeliac group, maximal at 8 hours which was not seen with corn enemas. In the control group an increase in IEL was also seen in response to wheat, but was not significant until 24 hours.

Loft et al (1989; 1990) used a peptic-tryptic digest of gluten to challenge a group of coeliacs and took biopsies from the rectum at frequent intervals over a four day period. Electron microscopy was used to examine lamina propria mast cells and computerised image analysis to quantify volumes of surface epithelium, crypt epithelium and lamina propria along with intraepithelial lymphocytes. The gluten digest induced significant lamina propria oedema which was biphasic in its time-course, infiltration of the lamina propria with lymphocytes and an increase in IEL compared to controls. Histological changes could be seen up to 96 hours after challenge. No response was seen to rectal challenge with β-lactoglobulin. The authors used an IEL response 6 hours after challenge of 10% or more to predict coeliac disease with sensitivity and specificity of approximately 90%. Immunohistochemical examination of the $CD3^+$ and $\gamma\delta^+$ populations within the mucosa may be even more sensitive (Marsh et al, 1993). Sturgess et al (1993) found a marked rise in $CD3^+$ IEL without significant change in the $\gamma\delta^+$ IEL in the early response to rectal gluten challenge suggesting that $\alpha\beta^+$ IEL are involved in the acute mucosal response to local gluten challenge.

Rectal challenging may prove to be a useful tool in the diagnosis of coeliac disease, especially in those individuals who do not present classically. De Sousa et al (1988), however failed to show any response to rectal gluten challenge in a group of children with untreated coeliac disease. In this study gluten enemas were given daily for eight days with biopsies taken at baseline and at the end of the challenge period. Measurements were made of total mucosal thickness, IEL counts, goblet to epithelial cell ratio, mitotic crypt cell activity and the degree of lamina propria cellular infiltration. The only measurement significantly different after gluten challenge was total mucosal thickness. The different challenging protocol in this study may account for the

absence of histological response of the rectal mucosa to gluten and highlights the need for a robust and reproducible method for rectal gluten challenging in order for it to be useful in routine clinical practice.

CHAPTER 2: STANDARD METHODS

The standard laboratory methods used in my work are described in brief in this section. Full laboratory protocols are presented in the Appendix.

Haematoxylin and Eosin Staining

The haematoxylin and eosin dye staining method is widely used in most histopathology laboratories as in most cases it enables a diagnostic decision to be made without the need for further specialised staining. Haematoxylin is acidophilic and so stains the acidic nuclei a blue-black colour, and eosin acts as a counterstain staining the cytoplasm and connective tissue various shades of pink, orange and red.

Haematoxylin itself does not stain the tissue, it must first be oxidised to haematein. This can be done naturally, by exposing the haematoxylin to light and air, but the process can take several months. Alternatively chemical oxidation using sodium iodate or mercuric oxide can be employed, and the dye is ready for use immediately. Haematein alone has poor affinity for tissue and so requires a mordant in order to bind to the section, and aluminium is used for this purpose in our laboratory. This stains the nuclei red initially, but changes to blue on washing with weak alkali. Eosins are xanthene dyes, and the most widely employed is eosin Y.

For staining, the sections are initially dewaxed in Histoclear and then hydrated through graded alcohol (absolute alcohol and then 70% alcohol) and tap water. They are then stained in alum-haematoxylin for 10 minutes followed by a 10 minute wash in tap water, which turns the staining blue. This blue staining of the connective tissues and cytoplasm is then removed by immersing in 70% alcohol. The sections are washed again in tap water and then saturated lithium carbonate to return the nuclear staining to blue. They are then counterstained in eosin for five minutes before a further wash

in tap water. Further dehydration through graded alcohols is then performed before a final immersion in histoclear and coverslipping in DPX.

Quantifying IEL in Small Intestinal Biopsies using Light Microscopy

IEL are usually increased in the small intestinal biopsies of patients with untreated coeliac disease or dermatitis herpetiformis. These cells can be demonstrated by haematoxylin and eosin staining, and in well orientated 3μm paraffin wax sections can be quantified when examined under the light microscope.

The microscope is set to give optimal resolution and even illumination by using Kohler illumination. The small bowel biopsy sections are examined using oil immersion and a x100 objective . Beginning at one edge of the slide and the bottom of a villus the epithelial cell nuclei and intraepithelial lymphocytes are counted. Lymphocytes straddling the basement membrane are included, but unidentifiable nuclear fragments are ignored. Areas of poor orientation or where the lamina propria has shrunk from the cell border are not counted, nor are goblet cells or crypt epithelial cells. In poorly orientated or small sections it is possible to count transversely cut villi, but a count may prove impossible in total villous atrophy. Cells are counted until 500 epithelial cells are reached and the IEL count expressed per 100 surface enterocyes. The normal range is 10-40 IEL per 100 surface enterocytes.

Staining of $CD3^+$ and $\gamma\delta^+$ Lymphocytes in Small Bowel Biopsy

The staining of these lymphocytes was performed by Mr John Bode. $CD3^+$ and $\gamma\delta^+$ lymphocytes are increased in the small intestinal epithelium of patients with coeliac disease. These cells can be detected by immunocytochemistry using the monoclonal antibodies, anti-CD3 and anti-$\gamma\delta$ on frozen biopsy sections enabling detection by microscopy.

Small bowel biopsies are orientated on embedding compound using a dissecting microscope prior to quick-freezing with Cryospray (Bright Instument Co, UK). They are then stored at -70°C until sectioning on a cryostat. 6μm sections are allowed to dry before fixing in acetone.

Fixed sections are incubated with normal rabbit serum (Scottish Antibody Production Unit (SAPU), Scotland) to block any non specific antibody interaction. Mouse anti-CD3 or anti-γδ (both SAPU) is then applied and incubated for one hour. The sections are washed before biotinylated rabbit anti mouse IgG (Dako Ltd, Denmark) is applied for a further hour and then washed off. Steptavidin/biodin peroxidase complex is then applied for another hour before rinsing. The sections are then treated with DAB before further washing with tap water. Haematoxylin is then applied, washed and lithium carbonate applied until the sections turn blue. They are then washed in tap water, 70% alcohol, absolute alcohol, isopropronal and histoclear before mounting in DPX.

Computerised Image Analysis

Image analysis is a method of making geometric and densitometric measurements on images from any source. Its main application is in quantitative microscopy, replacing traditional subjective measurements. The image analyser (Leica Q500MC, Leica, Germany) uses a television camera to sample an image from the microscope. The camera generates an electronic signal proportional to the intensity of illumination which is then digitalised into picture elements or "pixels". At each pixel the brightness of the image is sampled and this digital representation is analysed by the personal computer attached to the system.

$CD3^+$ and $\gamma\delta^+$ IEL Counts in Small Bowel biopsies

The surface of the epithelium is traced using the mouse and the stained cells marked and counted by the computer. Cumulative counts of several fields have been performed for each stain and the results shown in figures 2.1 and 2.2. It can be seen that for the $CD3^+$ counts ten fields should be counted to ensure an accurate measurement, but the $\gamma\delta^+$ cell counts require 20 fields to reduce variability in the count.

Antigliadin Antibody ELISA

This assay was performed by Mr John Bode. IgA- and IgG-class antigliadin antibodies are measured by a sandwich ELISA technique. Flat bottomed, gamma irradiated 96 well plates (Immulon 2, Dynatech Laboratories Inc, USA) are coated with a 25μg/ml gliadin solution (G-3375, Sigma Chemical Co, UK) before incubating at 22°C for five hours. The plates are washed three times before blocking with ELISA diluent (0.9% saline, 0.05% Tween 20 and 1% adult bovine serum) for two hours. Human standards and samples are diluted to 1/100 for IgA and 1/200 for IgG using diluent and added to the plates. The standard is applied in duplicate with doubling dilutions, and the samples are applied in duplicate in a single dilution. The plates are incubated overnight at 4°C in a moist, covered box. After further washing, alkaline phosphatase conjugated anti-human sera are applied before a further incubation at 22°C for five hours. Alkaline phosphatase substrate (Sigma) is applied after further washing and the colour allowed to develop. The plates are read using a Dynatech Microplate Reader using the top standard optical density of 1.00 as an endpoint. A standard curve is plotted and the sample values calculated from this.

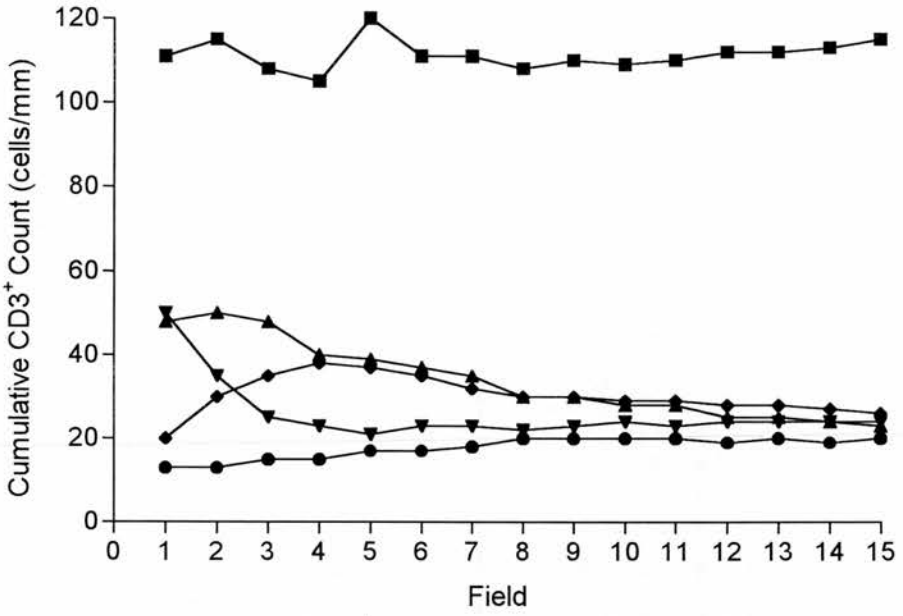


Figure 2.1: Cumulative $CD3^+$ cell count plotted against number of fields counted

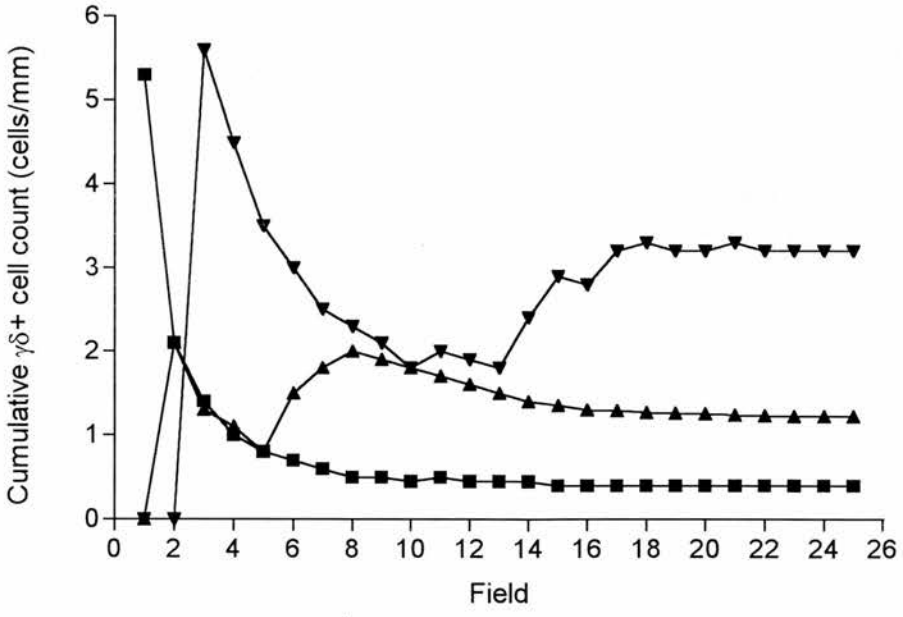


Figure 2.2: Cumulative $\gamma\delta^+$ cell count plotted against number of fields

Whole Gut Lavage Fluid Collection

The technique for performing whole gut lavage used in our department was developed by my predecessor, Dr Seamus O'Mahony. The technique is based on that described by Gaspari et al (1988), which in turn is adapted from a method originally developed for use in the mouse (Elson et al, 1984).

The solution used for the lavage procedure is a polyethylene glycol (PEG) based electrolyte solution. Commercially this is available as "Klean-Prep" or "Movicol" (Norgine, UK), and is widely used as a bowel preparation for barium enema or colonoscopy (DiPalma et al, 1984), and also for the treatment of severe constipation (Puxty and Fox, 1986; Logan et al, 1978). The commercial preparations are in the form of a sachet of powder which is mixed into one litre of water in order to make the solution.

Following an overnight fast, the lavage procedure begins at 8.30 am. The individual drinks the lavage solution at a rate of 250mls every 15 minutes until the material passed per rectum is a clear liquid, free of faecal material. Most individuals require approximately four litres to achieve this result. 200mls of this effluent are collected to be processed and aliquotted.

Whole Gut Lavage Fluid Processing and Storage

Initially, three 1.5ml aliquots of the raw fluid are taken and 6mg sodium azide added as a bactericidal agent. These tubes are labelled "unfiltered/unprocessed". 10ml of lavage fluid is filtered through a glass fibre filter (Whatman GF/A 12.5 filter paper). 5ml each of filtered and raw, unfiltered fluid are placed in separate tubes. To each is added, 500mg soya bean trypsin inhibitor (SBTI), 280μl 0.3M disodium ethylene diamine tetra-acetic acid (EDTA), 120μl of 0.3M PMSF (phenylmethylsulphonylfluoride) all of which act as protease inhibitors, and 12mg of sodium azide. The tubes are

allowed to stand for two minutes prior to the addition of 300μl newborn calf serum which provides an alternative substrate for any remaining protease (Elson et al, 1984). The fluid is then dispensed into microcentrifuge tubes and labelled "filtered/processed" and "unfiltered/processed" respectively. The tubes are stored at -70ºC prior to analysis.

Assay Techniques for PEG Based Rectal Wash Fluid

Albumin and Alpha-1-Antitrypsin

These assays were performed by Mr John Bode. When human albumin and α-1-antitrypsin react with their specific antibodies in the presence of polyethylene glycol, precipitating immune complexes form. In the presence of excess antibody these precipitates produce turbidity which can be photometrically measured at wavelength 340nm. This principle was used to measure the amounts of albumin and α-1-antitrypsin in the rectal wash fluid. Absorbance readings obtained by measuring calibration standards were used to generate a standard curve from which the concentration of albumin or α-1-antitrypsin in the sample was derived (Brydon et al, 1993).

These assays were performed on filtered/processed specimens using SPSO1 (Protein Reference Unit, Sheffield) as the calibration standard with sheep anti-human albumin and goat anti-human α-1-antitrypsin antibodies respectively. A quality control of gut lavage from a normal volunteer was used in which serum from the same volunteer was added to stimulate gut protein leakage and the results accepted when the quality control value was +/- 22% of the expected value. The results are expressed as μg of albumin or α-1-antitrypsin per ml of fluid.

Haemoglobin

This assay was performed by Mr John Bode. Haemoglobin was converted to fluorescing porphyrins by the removal of iron. Total haemoglobin was determined by reaction with heated oxalic acid:$FeSO_4$ reagent which converts haem to porphyrin without loss of preformed porphyrins. Fluorescence was measured using a luminescence spectrometer.

The analysis was performed on unfiltered/unprocessed material and the process of freezing causes haemolysis of any intact red blood cells allowing accurate measurement of total haemoglobin. Once thawed the samples were centrifuged to remove cell debris and the supernatant used.

Once the haemoglobin had reacted with the oxalic acid reagent a three step purification procedure was performed. The porphyrin analytes were extracted into an upper organic phase by adding potassium acetate and ethyl acetate/acetic acid. Butanol and potassium acetate in potassium hydroxide were then added to the organic phase in order to remove coproporphyrin and other porphyrins not derived from haemoglobin into a lower alkaline aqueous phase. Phosphoric acid/acetic acid was then added to the organic phase and the fluorescence of the lower acid extract was measured.

Cyanomethaemoglobin (Haemoglobin (Sigma Chemical Co Ltd, Uk) dissolved in Drabkin's reagent) was used as the standard and haemoglobin dissolved in Klean-Prep was used as the quality control. The coefficient of variation of the quality control material is currently 8%. The results were accepted if the quality control value fell within two standard deviations of the mean value of the quality control. Results are expressed is μg of haemoglobin per ml of fluid.

Total Immunoglobulins

These assays were performed by myself and Mr Kenneth Humphreys. Class A, G and M immunoglobulins were measured by ELISA. 96 well plates (Immulon 1, Dynatech Laboratories Inc., USA) were coated with goat anti-human IgA, G or M and incubated at 4°C overnight. The plates were washed three times before blocking with ELISA diluent and incubating at room temperature for one hour. Standard material (SPSO1 for IgG and IgM, colostrum for IgA) and filtered/processed samples were applied in doubling dilutions and the plates again incubated at 4°C overnight. The plates were washed three times before alkaline phosphatase conjugated goat anti-human IgA, G or M was applied. Following a three hour incubation period the plates were again washed and developing solution applied. The optical density of each well was then measured and a standard curve plotted.

The results were accepted if the correlation coefficient of the linear portion of the standard curve was > 0.985 and two points of the sample curves were parallel to the standard curve. Results are expressed as μg of immunoglobulin per ml of fluid.

Cytokines

These assays were performed by Dr Andrea Lear and Mrs Hazel Drummond. IL-1β, IL-6, IL-8 and TNF-α were assayed using commercial ELISA kits (R&D Systems, Eurogenetics and Cistron Biotechnology). Each kit used a quantitative sandwich ELISA technique. The plates were coated with a monoclonal antibody specific to the cytokine of interest. Samples and standards were pipetted into the wells and the cytokine bound by the immobilizing antibody. Unbound proteins were then washed away and an enzyme-linked polyclonal antibody specific to the cytokine applied. Following a further wash, a substrate solution was added to the wells and colour developed in proportion to the amount of bound cytokine. The colour intensity was then measured.

A standard curve was plotted and the sample values read off giving a concentration of cytokine (pg/ml of sample). Both filtered/processed and unfiltered/processed samples were used to assess whether filtration removed the cytokines from the samples.

Estimation of PEG concentration

This assay was performed by myself. Levels of PEG 3350 in the rectal wash fluid were measured by the method of Malawer et al (1967), a modification of the turbidimetric method of Hyden (1955). PEG 3350 was used as the standard and water as the blank. Filtered/processed samples were diluted to 1:10 with water before the addition to all the flasks of: Distilled water, $BaCl_2$, $Ba(OH)_2$ and $ZnSO_4$ with mixing between each stage. After standing for ten minutes the contents were filtered through double thickness filter paper (Whatman No 42).

1ml of filtrate was then added to 3ml of gum acacia and 4ml of 30% (w/v) trichloroacetic acid containing 5% (w/v) $BaCl_2$. The tubes were mixed and allowed to stand for one hour before the optical densities of the samples, standards and blanks were read using a spectrophotometer (Pye Unicam PU 8610) set at wavelength 650 nm. A standard curve was constructed and the samples extrapolated from this.

CHAPTER 3: USING COMPUTERISED IMAGE ANALYSIS TO QUANTIFY SMALL INTESTINAL INTRAEPITHELIAL LYMPHOCYTES

Introduction

Intraepithelial lymphocytes (IEL) have previously been quantified in small bowel biopsies by several different techniques. Ferguson and Murray (1971) described a differential count of the nuclei of the epithelial layer seen under light microscopy. Formalin fixed, paraffin embedded jejunal sections stained with haematoxylin and eosin (H+E) were used. The epithelium was examined under a high power objective enabling the nuclei of epithelial cells, lymphocytes and goblet cells to be distinguished and counted. Only well orientated areas of surface epithelium were used and small unclassifiable nuclear fragments were discounted. Goblet cell nuclei were not included, nor were intraepithelial eosinophils or lymphocytes in transit across the basal lamina. For each specimen a total of 500 epithelial cell nuclei were counted and the IEL count expressed as cells per 100 epithelial cells. The normal range using this technique was calculated to be 6-40 IEL per 100 villus epithelial cells.

An alternative method used a graticule within the eye piece of the microscope. This enabled a given length or area of epithelium to be examined. Formalin fixed, paraffin embedded sections were again used, and stained with H+E. Holmes et al (1974) examined 1660μm of epithelium and counted the nuclei of IEL contained within it. The count was expressed as IEL per mm epithelium. R Ferguson and colleagues (1974; 1975) used the graticule to divide the histological field into squares and nuclei within 50 squares of epithelium were counted and the total count converted into number of cells per mm^2 using a conversion factor. These techniques all showed an increase in the number of IEL in the small bowel biopsies of patients with coeliac disease and dermatitis herpetiformis.

Guix et al (1979) again used an eyepiece graticule, but this time measured a unit length of muscularis mucosa and from this calculated the epithelial surface area as well as the volume of the lamina propria. The IEL were counted in epithelium (villi and

crypts) related to the unit length of muscularis mucosa. In this case the count was given in absolute figures. This method showed no significant difference between IEL counts in coeliac and non-coeliac individuals, suggesting that the total number of IEL does not increase in coeliac disease, but reduction in the epithelial volume leads to an increase in the ratio of IEL to epithelial cells.

Increasingly, mild degrees of gluten sensitive enteropathy are being recognised with a raised number of IEL per 100 epithelial cells as the only abnormality. In view of the importance for my work of assigning patients to normal or abnormal groups with respect to mucosal infiltration by IEL, and as the ideal method for quantifying these cells would be by computerised image analysis, I decided to concentrate on IEL per unit length of mucosal epithelium.

Development of the Computerised Technique and Comparison with Light Microscopy

IEL were counted in 3μm sections of formalin fixed, paraffin embedded, H+E stained small bowel biopsies. These were either jejunal biopsies taken by Watson capsule under radiological control, or endoscopic distal duodenal biopsies, and the processing and staining of the sections were performed in the Hospital Pathology department. Further cutting and staining of sections was performed by John Bode, MLSO.

31 sections were analysed by computerised image analysis (Leica Q500MC described in Chapter 2 and x40 objective). Fields containing well orientated lengths of epithelium were chosen. Using the computer's mouse a line was drawn along the surface of the epithelium, following the contours as closely as possible. The computer is able to detect cells using pixel colour and I did hope it would be possible to exploit this facility in order to automatically detect the IEL. Unfortunately it proved impossible to set limits for pixel configuration and colour which were both sensitive and specific for IEL. I found that the nuclei of goblet cells exhibited almost identical colour staining and so were included in the count, and it also proved impossible to exclude the lamina propria

from the field, and so lamina propria lymphocytes were automatically detected too. In view of this I chose to manually select cells simply by "clicking" on them. The computer counted the selected cells in each field and kept a cumulative cell count for each biopsy. At the end of the count the computer then calculated an overall cell count expressed as IEL per millimetre of epithelium (IEL/mm).

Initially the number of fields required for an accurate IEL count was validated using five biopsies. Twenty fields (mean length of epithelium = 0.199mm) were analysed and cumulative count per millimetre of epithelium was plotted against number of fields analysed (figure 3.1). After eight fields the count plateaued and additional fields did not alter the count. Ten fields were used as a standard for further counts.

In our laboratory, IEL have previously been counted by light microscopy using the technique described by Ferguson and Murray (1971). This technique was therefore used as a standard comparison for the new computerised method. The same 31 sections were analysed by light microscopy (the method is described in detail in Chapter 2). Well orientated areas of epithelium were chosen and the nuclei of both IEL and epithelial cells counted (IEL per 100 epithelial cells).

Counts using the two techniques were compared (figure 3.2) and linear regression performed (r^2=0.964, $p<0.0001$). By extrapolation from this graph, and since a value of <40 IEL per 100 enterocytes has been the accepted reference range for light microscopy for 20 years, an interim upper normal limit of <53/mm was calculated. As high counts of IEL are of interest in gluten sensitivity I only defined the upper limit of the reference range, but if ever work on immune deficiency was carried out a separate lower limit would need to be calculated.

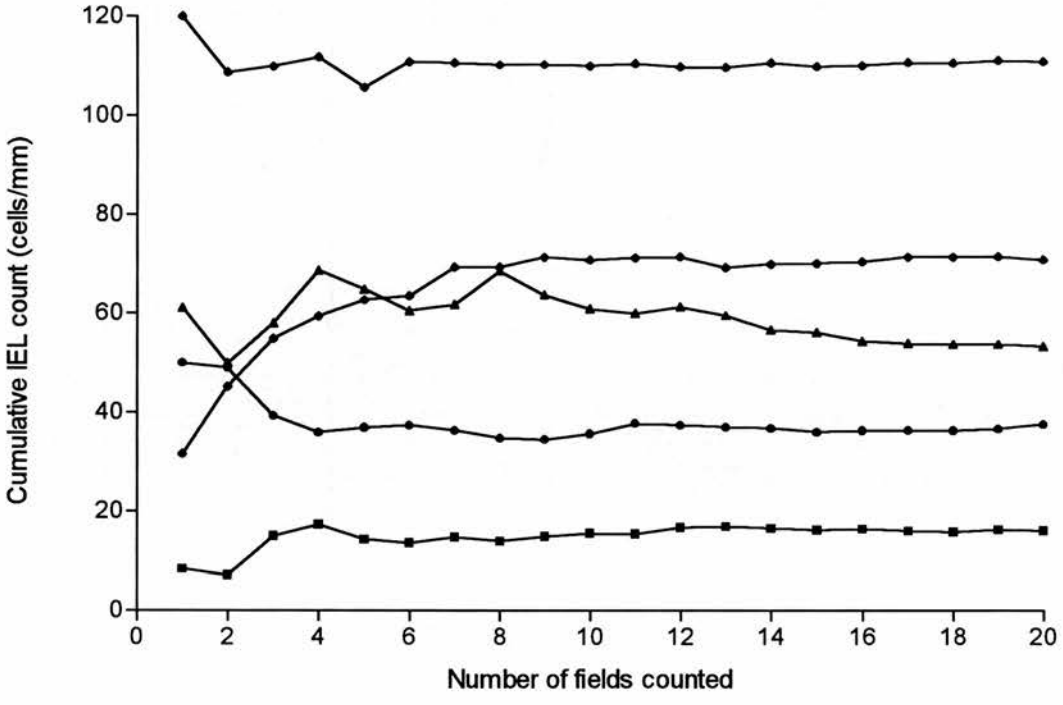


Figure 3.1: Cumulative IEL count by field in five small intestinal biopsies

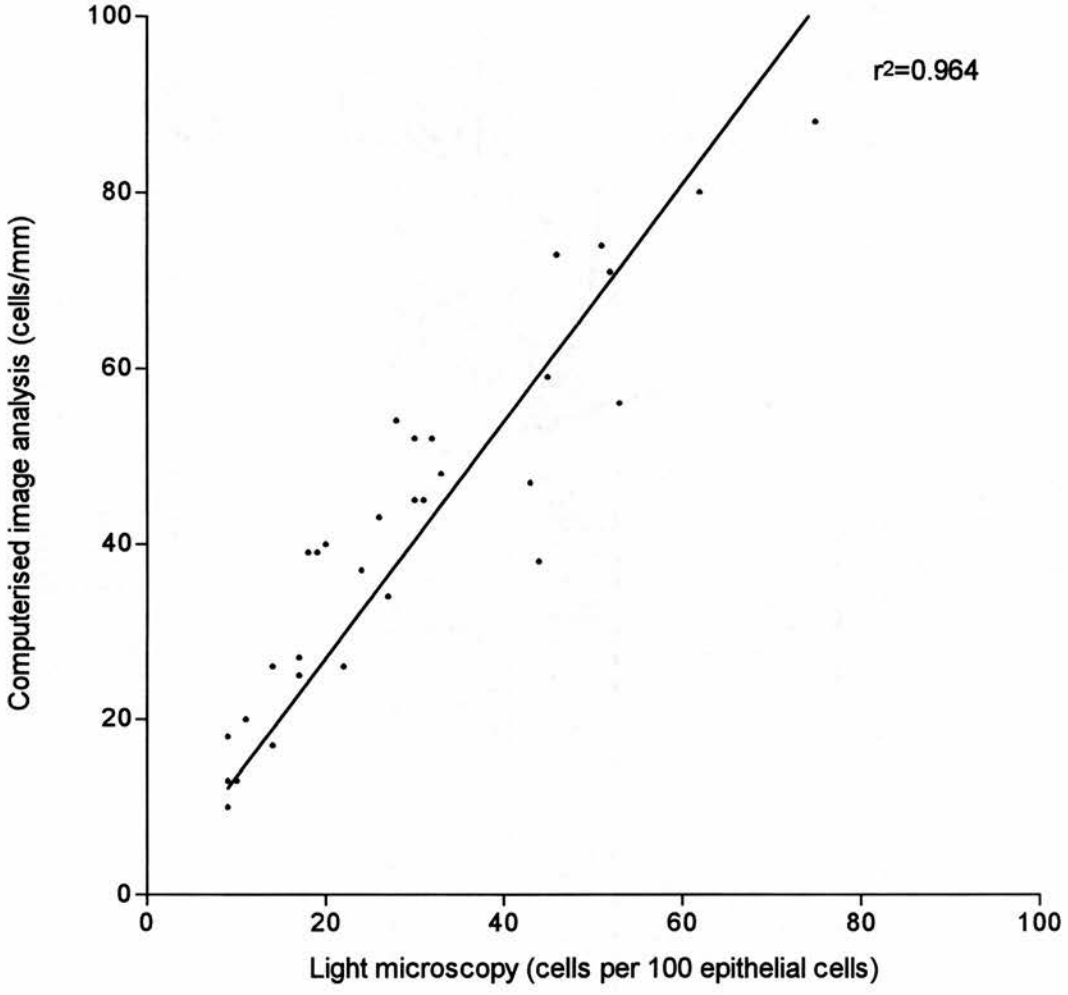


Figure 3.2: Comparison of IEL counts in small bowel biopsies using light microscopy and computerised image analysis.

Interbiopsy and Intrabiopsy Variability in IEL Count

I was concerned about the possibility of variability in the cell count depending on the site from which the biopsy was taken. In order to assess this variability counts were perfomed on archive material which had been collected over ten years earlier and stored. These were multiple jejunal biopsies taken using a multiple biopsy capsule. Two biopsies from each of nine patients were assessed. I also measured IEL in multiple sections taken from a single large jejunal biopsy to assess intra-biopsy variability. Three sections 100μm apart were used.

The results from the multiple biopsies are shown in table 3.1. The variability was not significant when analysed by the Wilcoxon Signed Rank test. The IEL counts from three sections taken from the same biopsy were 31.8/mm, 32.2/mm and 34.3/mm, therefore showing little variability.

	Biopsy No 1	Biopsy No 2
1	25.2	25.3
2	25.0	26.6
3	102.0	108.3
4	50.2	53.8
5	59.1	61.1
6	36.0	37.3
7	29.9	27.1
8	23.9	24.7
9	81.6	79.2

Table 3.1: Counts of two biopsies taken from each of nine patients using a multiple biopsy capsule. No significant difference between the counts (Wilcoxon signed rank test).

Interobserver Variability

For this technique to prove useful in a clinical laboratory setting it should not vary when used by different observers. In order to assess the inter-observer variability eight biopsies were examined by myself and Mr John Bode and the counts obtained are shown in table 3.2. There was no significant difference between observers when tested using the Wilcoxon signed rank test.

Biopsy	Observer 1 (HG)	Observer 2 (JB)
1	23	27
2	25	29
3	43	54
4	50	45
5	30	38
6	76	64
7	29	31
8	30	37

Table 3.2: IEL counts in eight small bowel biopsies performed by two independent observers

IEL Counts in Different Diagnostic Groups

In order to assess the differences in IEL counts in different disease states I counted IEL in small bowel biopsies from 82 patients in whom a gastrointestinal cause for their symptoms had been identified. These patients were divided into five diagnostic groups which are shown in table 3.3. The IEL counts are shown in figure 3.3 and were analysed by two sample T test.

There are significant differences between counts from coeliacs on normal diet and those on a gluten free diet ($p=0.021$), those with inflammatory bowel disease

(p=0.0052), those with irritable bowel syndrome (p=0.0094) and those with bile salt malabsorption (p=0.013).

There were also significant differences between the coeliac (gluten free diet) group and the IBD group (p=0.019) and the IBS group (p=0.020). The difference between the gluten free diet and bile salt malabsorption groups is not significant (p=0.11).

Patients with a final diagnosis of irritable bowel syndrome or bile salt malabsorption would be anticipated to have normal gut immunity and so can be used to assess the accuracy of the interim normal upper limit of up to 53 IEL/mm epithelium. It can be seen from figure 3.3 that counts in these groups generally fall below 50/mm whereas untreated and many of the treated coeliacs have counts greater than this value. An upper limit of 50 cells/mm was therefore used as a reference range.

Disease Group	C-ND	C-GFD	IBD	IBS	BSM
Mean age (years)	38.9	38.9	39.3	34.0	46.3
No of biopsies	13	15	7	20	28
Mean IEL count (per mm)	71.5	37.1	22.3	25.4	28.8
Standard Deviation	21.4	15.2	10.5	10.8	12.9
SEM	9.6	4.1	5.0	2.1	2.9
Range	44-124	16-66	13-43	10-35	9-59

Table 3.3: Disease groups in which IEL counts performed. C-ND = coeliac on normal diet, C-GFD = coeliac on gluten free diet, IBD = inflammatory bowel disease, IBS = irritable bowel syndrome, BSM = bile salt malabsorption, SEM = standard error of the mean

Conclusion

Using computerised image analysis to quantify IEL compares well to counting IEL by light microscopy and relating the total to the number of epithlelial cells. There is little variation within a single small bowel biopsy and also between biopsies taken from different sites within the upper small bowel. In addition, the technique is not operator dependent. In conclusion, therefore, computerised image analysis provides a reliable and robust technique for quantifying IEL.

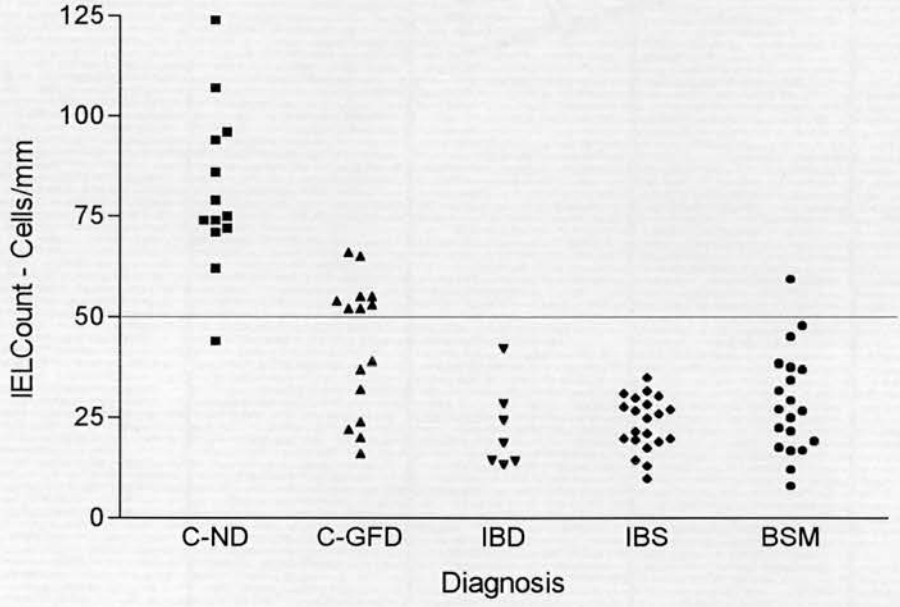


Figure 3.3: IEL counts on 82 biopsies from patients with various gastrointestinal diseases. C-ND = Coeliac on normal diet, C-GFD = Coeliac on gluten free diet, IBD = inflammatory bowel disease, IBS = irritable bowel syndrome, BSM = bile salt malabsorption. Horizontal line marks the interim normal reference range

IEL counts in biopsies with normal villous morphology

Pathology reports now often comment on the numbers of intraepithelial lymphocytes present in small bowel biopsies. Usually this is a subjective observation rather than a formal count of the cells. I looked at 34 biopsies with normal villous morphology in which the pathologist felt the IEL count was raised and 216 biopsies which were reported as being entirely normal.

In the normal group twelve patients had IEL counts of 50/mm or greater, but the remainder had counts within the normal range. In the group reported as showing increased IEL, 18 had counts of 50/mm or greater and 16 had normal counts. This suggests, therefore, that the subjective assessment of IEL counts is very difficult and inaccurate, and that in our hospital increased numbers of IEL are probably over reported. This highlights the importance of a formal, objective measurement, especially in those biopsies felt to have high numbers of IEL.

In view of the possibility of raised IEL counts representing low grade pathology gluten sensitive enteropathy, symptomatic patients seen in our gastrointestinal clinic have been offered a trial of gluten free diet to see if an improvement in symptoms could be obtained. In our group of 30 patients with normal morphology but increased IEL, seven were individuals with coeliac disease on gluten free diet and the details of the remaining 23 patients are listed in table 3.4.

The response of the first eight patients would suggest that these cases were indeed low grade pathology coeliacs, and this confirms the findings of my predecessor, Dr Eduardo Arranz. The patient who did not respond to the diet and those in whom symptoms were self-limiting may well have been cases of gastrointestinal infection. Ultimately these are the cases in which additional information, such as the presence of antiendomysium antibodies will be vital and where rectal gluten challenge could be useful.

Patient	Symptoms	IEL/mm	Treatment and Outcome
LM	Iron deficiency anaemia	64	GFD - anaemia now resolved
RM	Diarrhoea	62	GFD - diarrhoea resolved
KT	Diarrhoea	56	GFD - dairrhoea resolved
VC	Diarrhoea, lethargy	56	GFD - symptoms resolved
GF	Diarrhoea	52	GFD - diarrhoea resolved
KM	Diarrhoea	58	GFD - diarrhoea resolved
MB	Iron deficiency anaemia	50	GFD - anaemia resolved
FA	Diarrhoea	52	GFD - diarrhoea resolved
CR	Diarrhoea	52	GFD - no improvement
MB	Diarrhoea	54	Refused GFD
AJ	Iron deficiency anaemia	66	Refused GFD, given iron
JT	Folate deficiency	52	Refused GFD, oral folic acid
DR	Iron deficiency anaemia	54	Oral iron, not offered GFD
EM	Diarrhoea	52	Self-limiting
CP	Diarrhoea	72	Self-limiting
PT	Diarrhoea	63	Self-limiting
DM	Diarrhoea	53	Self-limiting
FM	Diarrhoea	53	Self-limiting
AJ	Weight loss	65	Self-limiting
NR	Diarrhoea	60	Self-limiting
JB	Diarrhoea	55	Self-limiting
BK	Diarrhoea	50	Bacterial colonisation - improvement with antibiotics
MG	Diarrhoea	59	Cholestyramine for bile salt malabsorption

Table 3.4: Clinical details of 23 patients with normal villous morphology and raised IEL counts

CHAPTER 4: IgA ENDOMYSIUM ANTIBODY MEASUREMENT

Introduction

IgA endomysium antibodies were first described in coeliac disease and dermatitis herpetiformis by Chorzelski et al (1983; 1984). They found that serum from these patients produced a fluorescent pattern within the smooth muscle of distal oesophagus from monkey when an anti-human FITC labelled antibody was applied. Similar fluorescence is also seen if human oesophagus is used (Uibo et al, 1995). Recently a new technique has been described replacing the oesophagus with human umbilical cord and examining for fluorescence within the smooth muscle of the cord vessel walls (Ladinser et al, 1994).

As I have previously mentioned, IgA endomysium antibodies demonstrate almost 100% sensitivity and specificity for coeliac disease. The sensitivity in dermatitis herpetiformis, however is much less, only 65% of patients with DH who are taking a normal diet will demonstrate this antibody (Volta et al, 1992). Nevertheless, the greater accuracy of this antibody over antigliadin antibodies has great potential within routine clinical practice. Antibody measurement provides a minimally invasive first line investigation for patients presenting with gastrointestinal symptoms which may be due to coeliac disease, whereas small intestinal biopsy, either by capsule or endoscopy is both invasive and time-consuming. These factors often lead to biopsy being offered late in the line of investigation after many other, sometimes more invasive investigations have failed to elicit a cause for the patient's symptoms.

Antibody measurement also offer an excellent means of screening individuals at high risk of coeliac disease, for example those with type I diabetes, Down's Syndrome or first degree relatives of coeliacs. Often these individuals are asymptomatic or have mild symptoms that are ignored both by the individual or health professionals. Despite this, however, evidence of malabsorption may be detected (Mäki et al, 1984a; Page et al, 1994; Sategna-Guidetti et al, 1994). In patients with symptoms from coeliac

disease it is known that many complications can develop if the diagnosis or treatment are delayed, or if treatment is incomplete (Holmes, 1996; Wright, 1995) but it is unclear if asymptomatic individuals are also at risk of these complications. Screening individuals at high risk of coeliac disease, therefore offers not only the opportunity to correct nutritional deficiencies before symptoms develop, but will also provide vital information as to the natural history of this group of patients.

As stated in the general introduction to this thesis, the antigen against which endomysium antibodies are directed is, as yet, unidentified. It is also unclear as to whether the antibody is generated in the intestinal mucosa or peripherally. Antigliadin and antireticulin antibodies have been found in intestinal secretions (Volta et al, 1988; O'Mahony et al, 1991; Mawhinney and Love, 1975) and so we were interested to examine whole gut lavage fluid for endomysium antibodies, as their presence would support mucosal production. For these reasons we were interested in developing the IgA endomysium antibody assay in Edinburgh, for both Health Service and research use.

It is of course important to remember that selective IgA deficiency is associated with coeliac disease (Collin et al, 1992b; Crabbé and Heremans, 1967; Beutner et al, 1989; Mawhinney and Tomkin, 1971; Walker-Smith, 1971) and that tests for serum IgA antibody are of no use in the detection of coeliac disease in these individuals. IgG reticulin antibodies have been found in 94% of those with gluten sensitive enteropathy and IgA deficiency (Collin et al, 1992b), whereas this class of reticulin antibody is found in only 59% of non-IgA deficient patients (Mäki et al, 1984b). IgG endomysium antibody are present in only 15% of coeliacs with normal IgA levels (Beutner et al, 1989), but Beutner et al (1989) described an IgA deficient patient with coeliac disease in whom IgG endomysium antibodies were present. I therefore modified the IgA EmA test to develop a technique for detecting the IgG-class of antibody which I would then have available if any patients of interest were IgA deficient.

This work was carried out by myself and Mr John Bode.

Development of the IgA Endomysium Antibody Assay

We developed the technique of indirect immunofluorescence against human umbilical cord based on that described by Ladinser et al (1994).

Fresh human umbilical cord was collected immediately after birth and cut transversely into 5mm blocks, embedded in embedding compound (Cyro-M-Bed, Bright Instrument Co Ltd, UK) and quick-frozen using Cryospray 22 (Bright Instrument Co Ltd, UK). The blocks were stored at -70°C, and appear not to deteriorate over many months. 8μm thick sections were cut using a cryostat at -15 to -20°C and placed on multispot slides coated with 0.1% poly-L-lysine (Sigma Chemical Co Ltd, UK). The sections were allowed to dry at room temperature for 30 minutes before wrapping the slides in aluminium foil and storing at -20°C. In order to assess how stable the sections were at this temperature, a batch of slides were kept for three months before being used with serum which had already tested positive. They did not deteriorate over this period of time.

After removing from the freezer the slides were allowed to reach room temperature whilst still wrapped in foil. This avoided the formation of condensation on the sections. Following a drying period at room temperature for 30 minutes the sections were fixed in a jar of dried acetone at -20° for 10 minutes followed by chloroform at room temperature for 30 minutes. The sections were then allowed to dry at room temperature for 30 minutes, and at this point the serum identification number was written on the top of the slide, and a circle was drawn around the section with a "PAP" pen (Dako Ltd, UK). From this point onwards the sections were not allowed to dry out again.

Initially we used phosphate buffered saline (PBS) with 0.05g bovine serum albumin (BSA) for making up serum and conjugate dilutions. We later found that ELISA diluent (0.09% saline, 0.05% Tween 20 and 1% adult bovine serum) had no adverse effects

on the assay, and using this diluent enabled us to perform both antigliadin and endomysium antibody assays on the same day, minimising the volume of serum required and conserving time.

After fixing, non-specific staining was blocked by covering the sections with diluent for 10 minutes at room temperature. The excess was then removed and 50μl of the serum was applied. Initially serum was applied without dilution, but this produced a great deal of background staining which interfered with reading the sections. Several dilutions were tried in order to find the optimum, and we finally agreed with Ladinser et al and used a dilution of 1:5. The sections were incubated at room temperature for an hour before rinsing with PBS and placing in a jar of PBS for 10 minutes on a rocking table. After drying around the sections to reduce contamination of adjacent sections, 50μl of diluted conjugate was applied to the sections. We used FITC labelled rabbit anti-human IgA (Scottish Antibody Production Unit, UK). Initially a dilution of 1:10 was used, and again this produced a great deal of background staining. A dilution of 1:50 produced only faint fluorescence with positive sera, and so an optimum dilution of 1:20 was used in subsequent assays. The sections were incubated for one hour in a dark staining box before placing in a jar of PBS and rinsed with running water for 10 minutes. The slides were again dried around the sections and mounted onto cover-slip with warm glycerol gelatin.

The sections were read in the early stages using a Wild M50 fluorescence microscope with a x10 objective. This microscope used a dark ground condenser on which a drop of immersion oil was applied. The slide was then placed on the condenser and read by two independent observers (myself and John Bode). The cord blood vessels were located and fluorescence looked for within the smooth muscle of the vessel walls. If fluorescence was found the sample was said to be positive for IgA endomysium antibody, and was negative in the absence of the fluorescence. The result was accepted as true if both observers agreed.

In May 1996 the laboratory purchased a new fluorescence microscopy (Hund H500, Hund, Germany) which does not use a dark ground condenser. The light intensity therefore does not decrease with a higher objective, enabling a greater appreciation of the pattern of fluorescent staining within the smooth muscle at x25 magnification (picture 4.1). Initially sections were read using both microscopes and no discrepancies in results were found between the two.

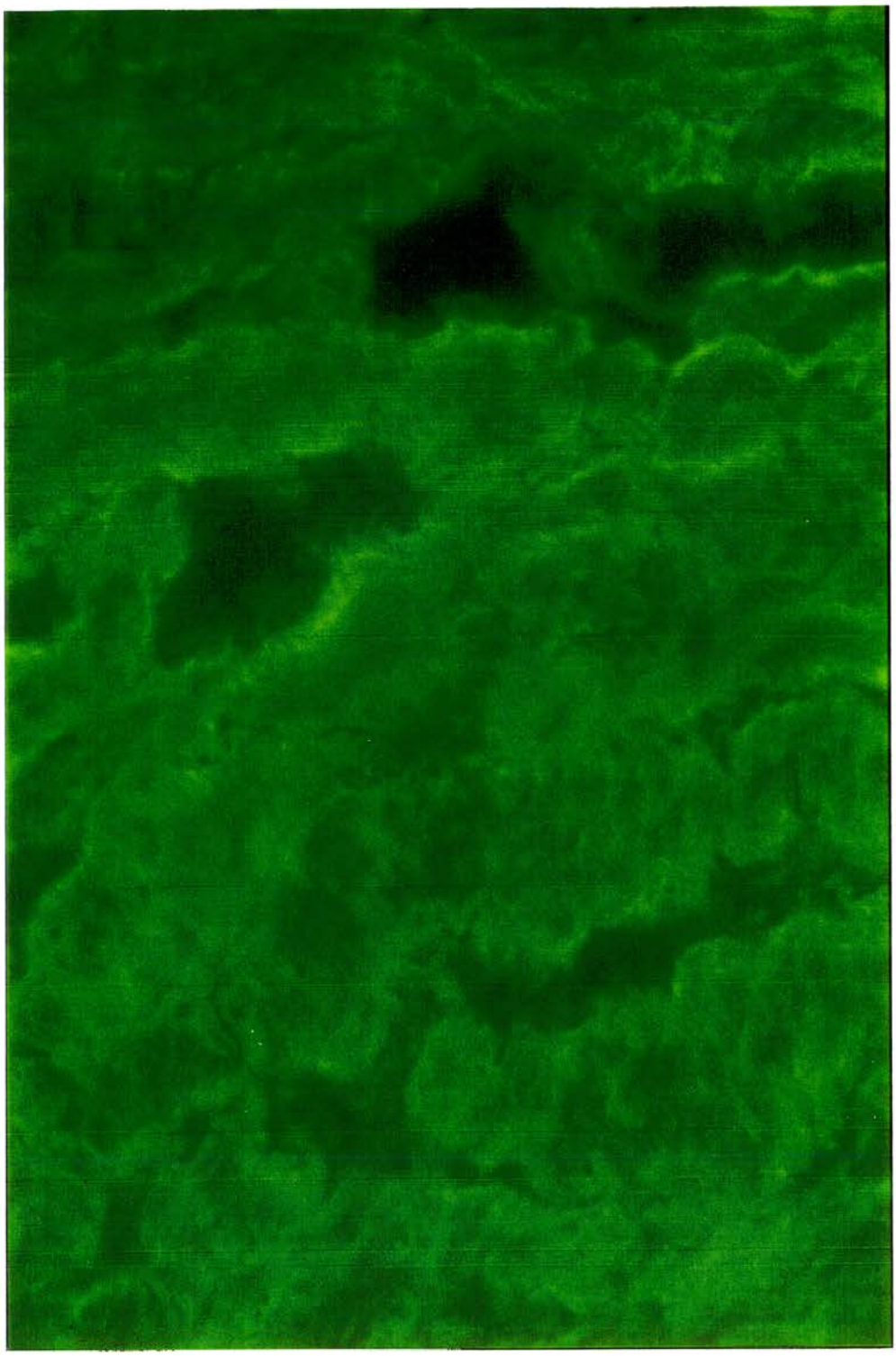
Validation of the IgA Endomysium Antibody Assay

The assay was performed on 97 serum samples to establish whether it could be added to the laboratory's NHS analytical service. Over several years serum has been stored on all patients undergoing jejunal biopsy and 51 of these historical samples were tested. In addition, 46 samples from patients undergoing investigation for possible coeliac disease were tested prospectively. All of these patients were taking a normal gluten containing diet and pathology reports of small bowel biopsy taken on the same day as the blood sample were available on all of them.

55 patients were confirmed to have coeliac disease. We found that of these, 48 were positive for IgA endomysium antibody, three were negative and four had equivocal results. Of the three with negative assays, two patients had selective IgA deficiency and one had dermatitis herpetiformis with subtotal villous atrophy on small bowel biopsy.

40 patients had entirely normal biopsies and 37 of these were negative for the antibody.

The two remaining patients were found to be positive for IgA endomysium antibody and their small bowel biopsies were reported as normal by the Pathologist. My predecessor, Dr Eduardo Arranz had performed formal IEL counts on these biopsies and had found them to be raised.



Picture 4.1: Typical fluorescence produced by endomysium antibody within the smooth muscle of the umbilical cord vessels

In order to calculate the sensitivity and specificity of the assay we excluded patients with equivocal assay results, IgA deficiency and those with isolated high IEL counts. The results are shown in table 4.1. The sensitivity was calculated to be 98% and the specificity 92.5%.

	Not Coeliac	Coeliac
Negative EmA	37	1
Positive EmA	3	48
Total	40	49

Table 4.1: Figures used to calculate sensitivity and specificity of the IgA endomysium assay in patients who had small bowel biopsy to establish the presence or absence of coeliac disease

Monitoring the Routine Use of the Assay

The IgA endomysium antibody assay was added to the laboratory's routine service on 1 January 1995. All samples submitted for antigliadin antibody measurement were also tested for endomysium antibody. After the first year of the service the results were reviewed.

In the twelve month period, 838 samples were analysed. 62 were positive for IgA endomysium antibody and the clinicians responsible for these patients were contacted to see if the patients had undergone small bowel biopsy. The ensuing feedback is demonstrated in table 4.2. 44 patients with negative antibodies also underwent biopsy and their results are shown in table 4.3. The formal IEL counts were performed by myself on these biopsies.

Biopsy Findings	Number of Patients
Normal	2
Increased IEL only	6
Partial villous atrophy	5
Sub-total villous atrophy	19
Total villous atrophy	3
Previously proven coeliac	16
Empirical GFD with clinical response, no small bowel biopsy performed	4
No biopsy performed	2
No reply from clinician	5

Table 4.2: Biopsy findings in 62 patients positive for IgA endomysium antibody

Biopsy Findings	Number of Patients
Normal	34
Increased IEL only	7
PVA (no improvement with GFD)	1
STVA and IgA deficiency	2

Table 4.3: Biopsy findings in 44 patients negative for IgA endomysium antibody

The patient negative for the antibody and with partial villous atrophy on biopsy was a man with Crohn's colitis and primary sclerosing cholangitis who was treated with azathioprine. His small bowel biopsy was not felt to be classical for coeliac disease but he underwent a trial of gluten free diet. He failed to respond to the withdrawal of gluten and was therefore classified as a non-coeliac individual.

In order to calculate the sensitivity and specificity of the assay over the first year of its use we excluded patients with isolated high IEL counts, those with IgA deficiency and those in whom biopsy had not been performed. The figures used for calculation are shown in table 4.4. The sensitivity was calculated at 100% and specificity at 94.6%.

	Not coeliac	**Coeliac**
Negative EmA	35	0
Positive EmA	2	43
Total	37	43

Table 4.4: Figures used to calculate specificity and sensitivity of the IgA endomysium antibody assay for patients who had serum sent for antibody testing over the first year of its routine use.

IgA Endomysium Antibody and Raised IEL Counts

In Chapter 3 I described 23 patients not known to have coeliac disease who had a raised IEL count as the only abnormality in their small bowel biopsy. Eight of these patients saw an improvement in symptoms on a gluten free diet, and three of this group had repeat duodenal biopsies in which the IEL count had returned to normal. In order to see if IgA endomysium antibody could predict individuals who would respond to this treatment I tested archived serum for the antibody. I tested serum from 20 patients, seven with self-limiting symptoms, eight in whom symptoms improved with a gluten-free diet, three who refused GFD, one who did not respond to the diet and one from a patient who responded to treatment for bile salt malabsorption. The results are shown in table 4.5.

Nearly 40% of the patients who responded to the diet but none of the those with self-limiting symptoms had IgA endomysium antibody. The individual who did not respond to the diet was negative for the antibody. This suggests that the presence of IgA endomysium antibody in such a patient will predict a response to GFD, but its absence does not predict a lack of response.

Patient	Outcome	IgA EmA
VC	Diarrhoea resolved and IEL count returned to normal on GFD	Positive
LM	Anaemia resolved and IEL count returned to normal on GFD	Positive
MB	Anaemia resolved on GFD	Positive
RM	Diarrhoea resolved and IEL count returned to normal on GFD	Negative
KM	Diarrhoea resolved and IEL count returned to normal on GFD	Negative
GF	Diarrhoea resolved on GFD	Negative
KT	Diarrhoea resolved on GFD	Negative
FA	Diarrhoea resolved on GFD	Negative
CR	No clinical improvement with GFD	Negative
MB	Refused GFD	Negative
JT	Refused GFD	Negative
AJ	Refused GFD - Anaemia treated with iron	Negative
CP	Self-limiting symptoms	Negative
JB	Self-limiting symptoms	Negative
DM	Self-limiting symptoms	Negative
FM	Self-limiting symptoms	Negative
PT	Self-limiting symptoms	Negative
EM	Self-limiting symptoms	Negative
NR	Self-limiting symptoms	Negative
MG	Diarrhoea resolved with cholestyramine	Negative

Table 4.5: IgA endomysium antibody in 20 individuals with raised IEL counts but normal small bowel mucosal architecture

IgA Endomysium Antibody in Whole Gut Lavage Fluid

Antigliadin and reticulin antibodies have been detected in intestinal secretions(Volta et al, 1988; O'Mahony et al, 1991; Mawhinney and Love, 1975) in coeliac disease and endomysium antibody has been found in organ culture medium after stimulation of small intestinal biopsy culture with gliadin. I therefore went on to examine whole gut lavage fluid for IgA EmA.

Samples

Whole gut lavage fluid (WGLF) had previously been collected and processed according to the standard methods described in Chapter 2 from nine patients known to have coeliac disease and one patient in whom IgA EmA was found whilst screening serum from a group of patients from the Gastrointestinal Unit. In addition, stored WGLF was tested from three patients who were non coeliacs. Serum had been collected at the same time as the lavage fluid and stored at -20°C.

Our standard technique was used for the measurement of IgA EmA in serum, and the only alteration made for WGLF was in the sample dilution. As WGLF is relatively dilute at the time of collection this was added to the umbilical cord sections without further dilution. The conjugate was added at 1:20 dilution as before and the sections read by two independent observers.

Results

IgA endomysium antibody was found in the serum of two patients, both on normal diets, and was also present in the lavage of both these individuals. A third patient, who was taking a gluten free diet, was negative for IgA EmA in serum, but was positive in lavage. The details are shown in table 4.6. The remainder of the patients were negative for the antibody in both serum and lavage.

Conclusion

In a recent paper McCord and Hall (1994) found IgA endomysium antibody in lavage in patients with dermatitis herpetiformis, but only in those individuals with the antibody present in serum. The finding of IgA EmA in lavage but not in serum in one of our patients on a gluten free diet further supports the view of mucosal production of this antibody. The titre may not be high enough to produce "spill over" into the peripheral blood, whereas this may well be the case in the two patients on normal diet with positive antibodies in both samples.

Patient	Diet	Serum IgA EmA	WGLF IgA EmA
PR	Normal	+++	+++++
IR	Normal	+++	+++++
MW	GFD	-	++++
AJ	GFD	-	-
EB	GFD	-	-
EK	GFD	-	-
JP	GFD	-	-
JD	GFD	-	-
MF	GFD	-	-
PB	GFD	-	-
JM	Non coeliac	-	-
EF	Non coeliac	-	-
	Non coeliac	-	-

Table 4.6: Serum and WGLF IgA endomysium antibody in nine coeliac, three non-coeliac patients and one patient found positive for IgA EmA on screening.

Development of the Method for Measuring IgG Endomysium Antibody

As I have mentioned, selective IgA deficiency is associated with coeliac disease, and those individuals with IgA deficiency have a tenfold risk of developing coeliac disease (Collin et al, 1992b). In view of this, we have adapted the method used for detecting IgA EmA for detecting the IgG-class of the antibody.

The frozen sections of human umbilical cord were fixed and blocked in the same way as with the IgA assay and diluted serum applied. Various dilutions of serum were tried, but a 1:10 dilution was found to be the most effective, reducing non-specific binding without the loss of accuracy. Following incubation at room temperature in a moist staining box for one hour the sections were rinsed with PBS and placed in a PBS bath for 10 minutes on a rocking table. FITC labelled rabbit anti-human IgG conjugate was then applied. A dilution of 1:20 increased the amount of background colouring, interfering with interpretation of the slides, and so a dilution of 1:40 was used. This improved the assay, but still produced prominent background staining. A conjugate dilution of 1:100 was found to be the most effective. After one hour's incubation at room temperature in a moist, dark staining box the sections were again washed and mounted onto coverslips with warm glycerol gelatin. The sections were read using a microscope fitted with UV light source, and were said to be positive if honeycomb fluorescence was seen within the smooth muscle of the cord vessel walls, and negative if this fluorescence was absent.

Results of the IgG Endomysium Antibody Assay

We initially used this assay to test the serum of a 44 year old woman with coeliac disease and abnormal serum immunoglobulins. She had intractable diarrhoea dating back to 1979, severe enough to cause the breakdown of her marriage. She underwent investigation at her local hospital and was found to have sub-total villous atrophy of the jejunal mucosa in the absence of antigliadin antibodies. There was a

reduction of total immunoglobulins of IgA-, IgG- and IgM-classes, but predominantly of the IgA-isotype.

She was referred to our hospital for further investigation as she failed to improve despite a strict gluten free diet and oral prednisolone. We measured her IgG-class coeliac related antibodies and found positive IgG endomysium antibody and a moderate titre of IgG antigliadin antibody (46U with the normal range of up to 45U). A repeat small bowel biopsy confirmed sub-total villous atrophy and intestinal permeability was raised when tested using a lactulose/rhamnose sugar permeability test. The remainder of her investigations, including whole gut lavage, stool culture and pancreatic function tests, were all normal.

She was treated initially with elemental diet with an improvement in her symptoms and the loss of serum IgG endomysium antibody. On withdrawal of the elemental diet her diarrhoea returned and so treatment with cyclosporin was commenced. This has produced good clinical and histological responses and her IgG endomysium antibody remains negative.

In this patient the presence of IgG endomysium antibody helped confirm the diagnosis of refractory coeliac disease and its subsequent disappearance on response to cyclosporin treatment has been used to monitor her clinical progress.

I tested 38 serum samples positive for IgA endomysium antibody for IgG EmA to assess how frequently both classes of the antibody occurred. Seven (18%) were positive for both classes of the antibody, but the remainder were negative for the IgG-class. Despite the small number of samples, this figure is in keeping with the findings of Beutner et al (1989).

49 samples negative for IgA EmA were also tested for the IgG-class antibody. Seven samples were positive for IgG EmA, all from individuals with IgA deficiency. Four were known to have coeliac disease, and the other three went on to have jejunal biopsies which revealed sub-total villous atrophy in each case.

It would appear, therefore, that the adaptation of the IgA endomysium antibody assay to detect the IgG-class of antibody provides a useful tool for the detection of coeliac disease in those individuals with IgA deficiency. IgG EmA occurs infrequently in non-IgA deficient individuals with coeliac disease making the test of little diagnostic use in these individuals. This lack of the IgG-class in those with normal IgA levels suggests recruitment of IgG plasma cells in the absence of IgA cells.

The Use of Dried Blood Spots in the Detection of Coeliac Disease

The use of serum or plasma for detecting coeliac related antibodies in large populations presents several different problems. The patient needs to have a formal venepuncture performed in order to obtain the blood, and not only is this a painful procedure for the individual, but also requires training of the member of staff performing the procedure. The blood needs to be collected into a suitable blood tube, sealed in a polythene bag, and then sent to the laboratory performing the assay. The sample must arrive in the laboratory within a few days to ensure that it has not deteriorated, and must then be centrifuged and the serum or plasma separated into a second sample tube which then requires freezing. The equipment required for the whole procedure and the delivery of the sample to the laboratory obviously have cost and manpower implications. The storage of large numbers of sera or plasma also presents problems providing suitable sub-zero storage space.

In view of these problems, we have looked at the possibility of using dried blood spots, obtained from a fingerprick, in the antigliadin and antiendomysium antibody assays. Dried blood spots are used extensively in newborn children for detecting inborn errors of metabolism, such as phenylketonuria and hypothyroidism. The advantages they offer over venepuncture samples for screening large populations are the relative inexpense of the equipment (a lancet for performing the finger or heel prick, and a compressed filter paper card), the less invasive method of obtaining the blood sample, the ease of sending multiple samples through the ordinary post if the distance from the

laboratory is great, the increased stability of the sample, the reduced storage space required and the lesser demands on manpower.

Previous work by Mrs Louise Handy, Mr Norman Anderson and Dr Nick Croft has looked at the possibility of measuring IgA and IgG antigliadin antibodies from dried blood spots using standard compressed filter paper cards. They have looked at the development of a technique for eluting serum from the spots and using the eluate in the antigliadin ELISA. This initial work looks very promising, with little variability between different spots taken from the same serum sample and excellent stability of the samples stored at room temperature and 4°C over many months. Mrs Handy went on to examine spots made from 40 whole blood samples and compared the antigliadin titres in the eluates to those in plasma from the same sample. The samples were eluted by a method based on that of Tappin et al (1991). A 5mm disc was punched from the centre of the spot and placed into a microtitre well containing 200µls of elution buffer (PBS, 0.05% Tween 20 and 0.05% sodium azide). The plate was shaken on an ELISA mixer for 30 minutes before and after an overnight incubation at 4°C. The eluate was then taken from the well and used in the antigliadin antibody ELISA assays at a dilution of 1:5. The titres were then compared to those in plasma by linear regression and correlation coefficients of r^2=0.576 and r^2=0.787 were calculated for the IgA and IgG assays respectively. These positive results led on to my development of a technique for measuring IgA endomysium antibody on similar material.

Development of the Method for Measuring IgA Endomysium Antibodies in Dried Blood Spots.

Paired whole blood and serum samples were collected from 12 patients attending the Gastrointestinal Unit for investigation; four with proven coeliac disease and eight non-coeliacs. Serum antiendomysium antibodies were measured by indirect immunofluorescence against human umbilical cord using the method described earlier.

In order to measure antiendomysium antibodies from dried blood spots the sections of cord were fixed and blocked in the same way. Initially the dried blood spots were eluted as described above and the eluate applied to the section of umbilical cord without dilution. The sections were incubated at room temperature for one hour prior to washing and application of the conjugate. This method did not produce positive results and I felt this was due to a combination of failing to elute all of the antibody from the spot and over-dilution of the material by using a volume of 200μls for elution. I therefore attempted to elute the spot directly onto the sample. After fixing and blocking, 75μl of a solution was applied to each section. A 5mm disc was punched out of the centre of the filter paper circle and "floated" on the liquid. The sections were then incubated overnight at 4°C. Initially I used the original endomysium assay diluent, namely PBS with bovine serum albumin, but found that the sections were either negative or equivocal but never positive. This was not improved by using ELISA diluent, but the use of elution buffer did improve the quality of the results. I found that better elution was achieved after shaking the slides on an ELISA mixer for 30 minutes prior to the overnight incubation. Before washing the next day the slides were again shaken for 30 minutes. The conjugate and coverslips were applied as before.

The sections were read blindly by two independent observers, looking for honeycomb fluorescence within the cord vessel smooth muscle. Results were accepted if both observers agreed.

The results of the twelve paired samples are shown in table 4.7. In all cases the results of the serum sample and the dried blood spot agreed. The eight non-coeliac patients were all negative for IgA endomysium antibody, and the four with coeliac disease were all positive for the antibody. The degree of positivity varied between serum and dried blood spot, but this is a very subjective measurement and is likely to be very inaccurate. In all cases, however, the antibody could be described as "strongly positive".

I found that positive staining within the vessel smooth muscle occurred in the same pattern as seen with serum, but strong fluorescence also occurred within the connective tissue around the vessels. It would appear that this connective tissue deteriorates overnight and holes develop within the tissue. Fluorescent staining then occurs at the edges of these holes, as if endomysium is exposed during this degenerative process.

	IgA antiendomysium antibody	
	Serum	**Dried blood spot**
Non- Coeliacs		
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	-	-
7	-	-
8	-	-
Coeliacs		
1	+++	++++
2	+++	+++
3	++++	+++
4	++++	++++

Table 4.7: Comparison of IgA endomysium antibody assay results in serum and dried blood spot samples from 12 patients

Summary

In summary, therefore, we have shown that it is possible to detect coeliac related antibodies in material eluted from dried blood spots. Initial studies revealed good correlation between the titres of antigliadin antibodies from the blood spots compared with plasma. Excellent correlation was also found between blood spot and serum results in the IgA endomysium antibody assay.

Obviously, these experiments were carried out on small numbers of samples, but the positive results are encouraging and work on these assays is continuing. The blood spots were also produced in ideal conditions, exactly 50μls of blood was pipetted onto each spot. This will not be the case in clinical practice and so future experiments should include material obtained by the patient him/herself to see if under-filled spots will affect the results of the assay. We also need to look at the amount of material eluted from the blood spots to see if this varies with different conditions.

I think these techniques show a great deal of promise for use as screening tools in the future. In newborns dried blood spots are used as an initial test for inborn errors of metabolism and positive or equivocal results are followed up by repeating the tests on venepuncture samples. I am sure this practice should also be followed in the case of coeliac disease screening by this method.

CHAPTER 5: THE USE OF ANTIGLIADIN AND ENDOMYSIUM ANTIBODIES IN SCREENING FOR COELIAC DISEASE

Introduction

As I have already mentioned, both antigliadin and tissue antibodies have been used to screen for coeliac disease in both normal and "at risk" populations (Friis and Gudmand-Hoyer, 1986; Uibo et al, 1993; Vazquez et al, 1996; Deseta et al, 1995; Ribes et al, 1991; Vitoria et al, 1994; Calero et al, 1996; Mäki et al, 1984a; Page et al, 1994; Sategna-Guidetti et al, 1994; Nosari et al, 1996). Very few studies however, have looked at hospital populations and so, once our endomysium antibody assay had been established, we screened a population of hospital patients for coeliac disease. Following on from this we went on to screen two insulin-dependent diabetic populations and a group of individuals with Down's syndrome.

HOSPITAL POPULATION

Materials and Methods

300 whole blood samples were obtained from the hospital Haematology laboratory. These samples had all been sent to the laboratory for full blood count estimation and the residual sample was used for coeliac disease screening. The whole blood was centrifuged at 2500 rpm for 10 minutes before separating the plasma, which was then stored at -20°C. In addition, 600 stored serum samples were also used. These had been collected from patients attending the Gastrointestinal Unit for investigation over the previous three years and had been stored at -20°C.

The samples were initially tested for antigliadin antibodies, plasma by Brian Walker and Louise Handy as part of a special study module and serum by Norman Anderson. Any samples with high IgA antigliadin antibody titres were also tested for IgA endomysium antibodies.

The physician responsible for sending any positive sample was contacted for patient details and further investigation for the patient offered.

Results

Haematology Samples

41 samples were found to have IgA antigliadin antibody titres over 30U and all were tested for IgA endomysium antibody. These results along with the clinical details given to the Haematology laboratory are shown in tables 5.1 and 5.2.

Eleven patients tested positive for IgA endomysium antibody, one of whom was already known to have coeliac disease. The remaining 27 samples were negative. There was no significant difference between the IgA antigliadin antibody titres in the two groups using the Mann-Whitney test.

Patient	Age	Clinical Details	AGA (U)	AEA
1	47	Alcoholic liver disease	47	Positive
2	84	Alcoholic liver disease	73	Positive
3	67	Alcoholic liver disease	87	Positive
4	51	Alcoholic liver disease	42	Positive
5	69	Neuropathy, alcoholic liver disease	106	Positive
6	55	Coeliac	31	Positive
7	80	"On warfarin"	54	Positive
8	95	Weight loss, diarrhoea	60	Positive
9	80	Pneumonia	97	Positive
10	77	Gastric carcinoma, pernicious anaemia	193	Positive
11	89	Anaemia	36	Positive

Table 5.1: Results and clinical details of patients in the haematology group with IgA antigliadin antibody titres of >30U and positive IgA endomysium antibody

Patient	Age	Clinical Details	AGA (U)	AEA
1	29	Crohn's disease	66	Negative
2	77	Cardiac Failure	40	Negative
3	64	Idiopathic thrombocytopenia	31	Negative
4		Post natal	40	Negative
5	75	Polymyalgia Rheumatica	35	Negative
6	76	"On iron"	35	Negative
7	67	Gout	46	Negative
8	65	Pre-operative	32	Negative
9	73	Sub-dural haematoma	98	Negative
10	68	Rheumatoid arthritis	33	Negative
11	62	Inflammatory bowel disease, arthritis	118	Negative
12	30	Pregnant	45	Negative
13	75	Diverticulitis	53	Negative
14	63	Diverticulitis, Ischaemic heart disease	53	Negative
15	71	Abdominal pain	54	Negative
16	63	Pre-operative	44	Negative
17	33	Cystic fibrosis, heart and lung transplant	58	Negative
18	49	Rheumatoid arthritis	41	Negative
19	58	Stroke, on warfarin	42	Negative
20	81	Type I diabetes, ?pulmonary embolism	37	Negative
21	65	Breathlessness	91	Negative
22	68	Rheumatoid arthritis	32	Negative
23	30	Rectal bleeding	88	Negative
24	83	Renal failure and oedema	42	Negative
25	81	Altered bowel habit	57	Negative
26	65	Myeloma	45	Negative
27	26	Chronic myeloid leukaemia	40	Negative

Table 5.2: Results and clinical details of patients in the haematology group with IgA antigliadin antibody titres of >30U and negative IgA endomysium antibody

Gastrointestinal Unit Samples

Of the 600 samples tested, 93 had high IgA antigliadin antibody titres and were tested for IgA endomysium antibody. 16 were positive for IgA EmA and their details are shown in table 5.3. Seven of these patients were already known to be coeliac, and one patient had dermatitis herpetiformis.

Patient	Diagnosis made in GI Clinic	AGA (U)
1	Bronchial carcinoma, diarrhoea	63
2	Bacterial colonisation	371
3	Depression, Irritable bowel syndrome	64
4	Alcoholic liver disease, angiodysplasia	68
5	Colonic carcinoma	30
6	Ulcerative colitis, Ischaemic heart disease	41
7	Bile salt malabsorption, Type II diabetes	33
8	Angiodysplasia, Iron deficiency anaemia	30
9	Dermatitis herpetiformis	31
10	Coeliac Disease	>400
11	Coeliac Disease	>400
12	Coeliac Disease	109
13	Coeliac Disease	>400
14	Coeliac Disease	41
15	Coeliac Disease	142
16	Coeliac Disease	>400

Table 5.3: Clinical details and IgA antigliadin antibody titres of GI patients found positive for IgA endomysium antibody

Follow up of Patients with Positive Results

In total there were 18 patients positive for both antibodies from the two groups who were not known to have coeliac disease or DH. Five of the patients from the Haematology group and one from the GI group had alcoholic liver disease. Of these two had died soon after the sample had been collected and one was too unwell to have further investigations. The remaining three patients all underwent endoscopic duodenal biopsy and in each case the mucosa was normal.

The follow up of the remaining 12 patients is shown in table 5.4:

Clinical Details	No of Patients	Outcome
Diarrhoea, thryroid disease	1	Small bowel biopsy confirmed coeliac disease
Ulcerative colitis	1	No biopsy taken
Diarrhoea	2	Resolved with drug therapy
Iron deficiency anaemia	1	Refused investigation
Angiodysplasia, on warfarin	1	Not biopsied in view of anticoagulation
Malignancy	3	Died
No reply from Physician	3	

Table 5.4: Outcome of follow up of 12 patients from the two groups with positive IgA EmA and raised IgA antigliadin antibody

Summary

From our original pool of over 900 samples we found one new case of coeliac disease. The data illustrate the difficulties encountered in screening a population for coeliac disease. Many of the patients were elderly and often had several co-existing illnesses making further investigation difficult. As many of the samples were several months or even years old by the time they were tested patients had been lost to follow up or had died.

I was surprised by the high number of patients with alcoholic liver disease who were positive for IgA EmA, especially as the three who were biopsied had normal small bowel morphology and IEL counts. I therefore went on to investigate the possibility of alcoholic liver disease producing a false positive endomysium assay, and this work is presented in Chapter 6.

INSULIN-DEPENDENT DIABETIC POPULATION

Increasingly it is recognised that insulin-dependent diabetes mellitus (IDDM) and coeliac disease are associated. Several studies have screened insulin-dependent diabetics using antigliadin antibodies and have found the prevalence of coeliac disease to be between 1.7 and 16% (De Vitis et al, 1996; Boudraa et al, 1996; Collin et al, 1989; Barera et al, 1991; Page et al, 1994) and using reticulin antibodies puts the prevalence at 1.4-3.8% (Mäki et al, 1995; Saukkonen et al, 1996; Savilahti et al, 1986; Mäki et al, 1984a). More recently IgA endomysium antibody has been used for screening and has found coeliac disease in 3.13-6.4% of type I diabetics (Rensch et al, 1996; Sategna-Guidetti et al, 1994; Nosari et al, 1996).

We have looked at two groups of patients with insulin-dependent diabetes, adults attending the Edinburgh Royal Infirmary and children attending the Royal Hospital for Sick Children, using a combination of IgA antigliadin and endomysium antibodies.

Sample Collection

Adults

Blood was taken from all patients with type I diabetes attending the diabetic clinic between March 1996 and January 1997 by the clinic nursing staff. In total 607 samples were received, and all were centrifuged and serum separated before storing at -20°C prior to analysing.

Children

All the children and their parents attending the paediatric diabetic clinic were approached between August 1996 and January 1997. They were given written and verbal information regarding the association between IDDM and coeliac disease.

In Edinburgh the monitoring of children with diabetes is primarily using capillary blood obtained by fingerprick to measure glycosylated haemoglobin (HbA_1C) and annual thyroid function tests. In view of this I decided to use capillary plasma to measure coeliac antibodies in order to avoid venepuncture.

Children having blood taken in the clinic were asked if I could collect an additional sample at the same time, and those not having blood were asked to send in a sample collected at home. For the home collection kit I included a filter paper card in addition to the capillary tube and asked that both be used in order to ensure a sufficient quantity of blood was received.

Capillary blood was collected into a Microvette CB300 lithium heparin tube (Sarstedt, Germany). Once received in the laboratory the samples were centrifuged at 2500 rpm for five minutes and the plasma separated and stored at -20°C prior to testing. Dried blood spot samples were stored in a polythene bag at 4°C. In total 130 samples were received from the 161 children approached (80.1%).

Methods

All samples were tested for IgA antigliadin and endomysium antibodies using the techniques previously described. Serum was tested at 1:5 dilution and plasma at 1:10 dilution for the endomysium assay, but all were tested at 1:100 dilution for the antigliadin assay.

Any patients with positive endomysium antibodies were contacted and offered an outpatient appointment to discuss the implications of the positive results before further investigations were arranged.

Results

IgA endomysium was found in 17 adult samples and one paediatric sample. All the patients and their general practitioners were contacted and offered further investigation. One patient did not attend her clinic appointment. The clinical details of the remaining 17 patients are shown in table 5.5. Five patients were completely asymptomatic but of these, only one had completely normal haematological and biochemical parameters. Twelve patients did have symptoms, ranging from mild abdominal bloating to severe diarrhoea and vomiting. One man, aged 77, had iron deficiency anaemia and coexisting pernicious anaemia, but also suffers from haemophilia A and so was not offered biopsy. He was advised to reduce his gluten intake as much as possible in addition to his iron supplementation. The patient found from the paediatric clinic also had severe symptoms of diarrhoea, vomiting and abdominal pain leading to many lost school days. She was failing to gain weight and her diabetic control has been very poor over the last two years. Her symptoms have improved on a gluten free diet and she is now gaining weight.

The commonest biochemical abnormality was hypomagnesaemia, found in ten of our 17 patients. I was surprised by this finding, as hypomagnesaemia is relatively uncommon in our clinical experience of coeliac disease. I wonder if this is due to increased renal losses of magnesium occurring in diabetes and the inability of the bowel to compensate for this by increasing absorption. I have not looked for a correlation between these results and the finding of microalbuminuria, but this would be worthwhile. Hypocalcaemia was not seen in any of our patients. Thyroid antibodies were found at an equivocal titre in three asymptomatic female patients, but no other organ specific autoantibodies were found.

At the time of submission of this thesis, biopsy findings are outstanding in most of the patients, but are available in four. Only one patient had findings of classical coeliac disease with villous atrophy, crypt hyperplasia and increased IEL. One patient was reported to have an entirely normal biopsy, and an IEL count was performed which was normal. A second biopsy was taken for crypt microdissection by Dr Quaioling Liang and this showed shortening of the villi, lengthening of the crypts and an increased in the number of mitotic figures within the crypts. Two patients were reported to have increased numbers of IEL and formal counts of these biopsies are outstanding. These biopsies also showed patchy crypt lengthening on micro-dissection. Counts of $CD3^+$ and $\gamma\delta^+$ IEL are still awaited on all these biopsies.

Summary

Testing a general hospital population failed to find any new cases of coeliac disease, but did lead to the finding of high IgA antigliadin titres and positive IgA endomysium antibodies in a number of patients with chronic liver disease. One patient who had attended the gastrointestinal clinic several years ago was confirmed to have coeliac disease and a second patient with inflammatory bowel disease is awaiting endoscopic biopsy to confirm the diagnosis as a result of this testing.

In the insulin-dependent diabetic population, 17 adults and one child were found to have endomysium antibody and an unexpectedly high number were found to have gastrointestinal symptoms. One patient has typical histological features of coeliac disease and three have findings suggestive of gluten sensitivity. All these patients have received dietetic advice regarding a gluten free diabetic diet and their progress will be reviewed in the clinic.

Our findings support the view that antigliadin and endomysium antibodies should be tested routinely in diabetic clinics to identify coexisting coeliac disease which may result in significant morbidity in these patients.

Patient	Age	Symptoms	Haematology/Biochemistry	Biopsy Findings
WA	77	Tiredness	Iron deficiency anaemia, hypomagnesaemia	Not done
CC	26	Diarrhoea	Normal	Crypt lengthening, IEL count 51/mm
MC	16	Diarrhoea, vomiting	Hypomagnesaemia	Villus shortening, crypt hyperplasia, IEL 25/mm
AC	18	None	Hypomagnesaemia	Awaited
MD	41	Diarrhoea	Iron deficiency, hypo-magnesaemia	Severe PVA
HE	50	Bloating	Iron deficiency	Awaited
AH	33	Diarrhoea, vomiting	Hypomagnesaemia	Awaited
HH	39	Vomiting	Hypomagnesaemia	Awaited
AJ	66	None	Iron deficiency, hypo-magnesaemia	Declined
BM	34	None	Hypomagnesaemia	Declined
KM	21	None	Hypomagnesaemia	Awaited
RM	35	Diarrhoea	None	Crypt lengthening, IEL count 35/mm
EM	66	None	Normal	Declined
NS	23	Diarrhoea	Hypomagnesaemia	Declined
KS	35	Bloating	None	Awaited
JS	44	Diarrhoea, vomiting	None	Awaited
RW	43	Tiredness, diarrhoea	Iron deficiency anaemia	Awaited

Table 5.5: Clinical details of 18 type I diabetics with positive IgA endomysium antibody on screening.

CHAPTER 6: ANTIGLIADIN AND ENDOMYSIUM ANTIBODIES IN CHRONIC LIVER DISEASE

Introduction

Coeliac disease and primary biliary cirrhosis are known to be associated (Logan et al, 1978; Olsson et al, 1982). Lindgren et al (1994) have reported a higher incidence of coeliac disease in a group of patients with cryptogenic cirrhosis but no other discrete liver disease has been found in association with gluten sensitivity. I was therefore surprised by the finding of positive IgA endomysium antibody in serum and plasma from patients with alcoholic liver disease. Three of our six positive patients underwent small intestinal biopsy which was normal in each case. This raised the possibility of chronic liver disease producing a false positive result in our IgA EmA assay. I went on to test 25 serum samples for this antibody from patients with chronic liver disease.

Materials and Methods

25 serum samples were obtained from individuals with chronic liver disease, both from our own Unit and also from Dr Ruth Gillespie at the Royal Infirmary's Gastrointestinal Unit. The diagnoses of these patients are listed in table 6.1. These samples were tested for IgA antigliadin and endomysium antibodies.

Samples found to be positive for IgA EmA were assessed further by repeating the endomysium assay at increasing dilutions to measure titres of the antibody. In addition, total IgA levels were also measured by the hospital Biochemistry laboratory.

Results

Eight samples had raised IgA AGA titres and ten were positive for IgA EmA (table 6.1). Four individuals agreed to undergo small intestinal biopsies, and all were entirely

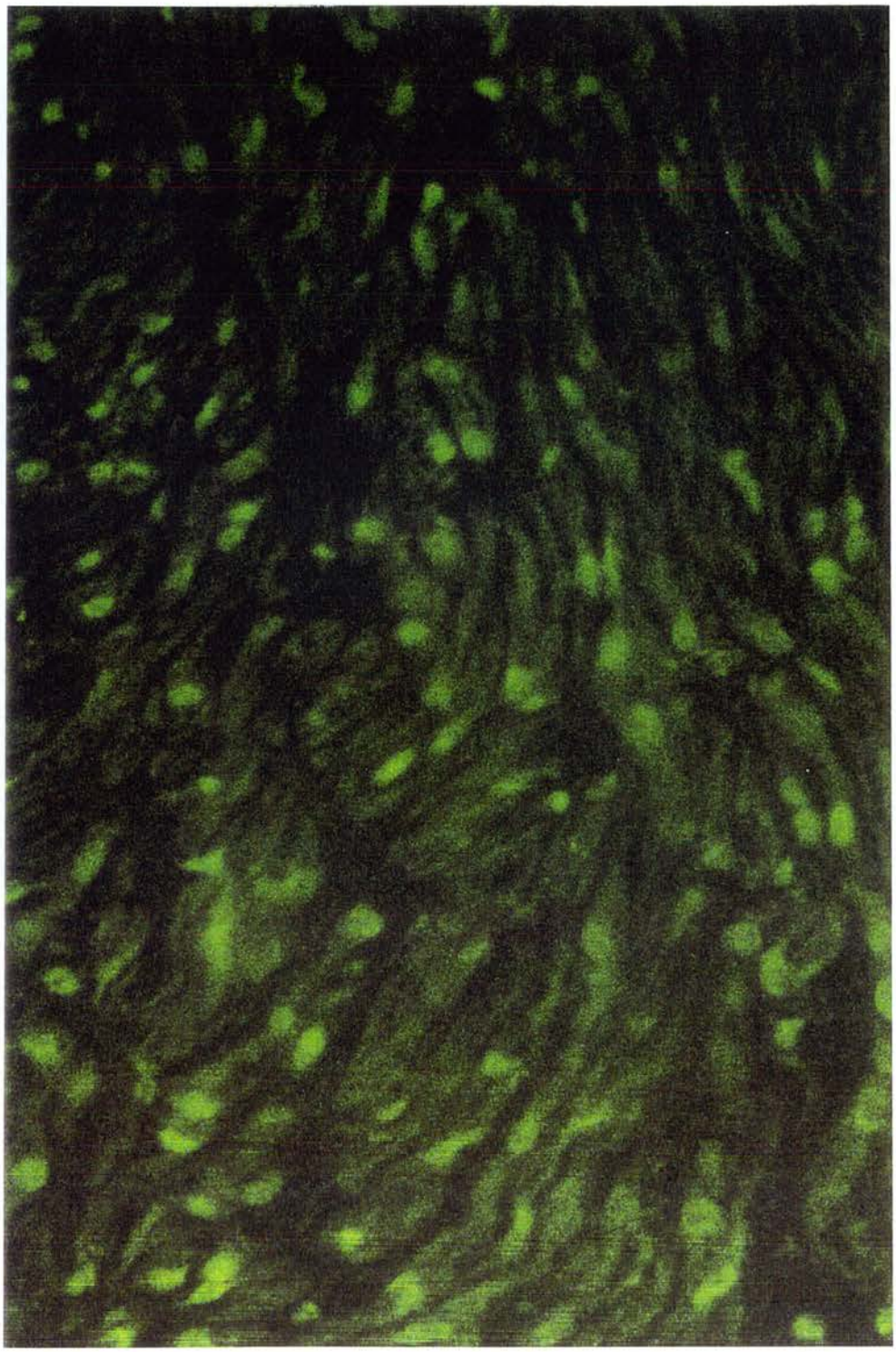
normal. Nine out of ten with positive IgA endomysium antibodies had raised total IgA levels.

Diagnosis	No. of Patients	No. with raised AGA	No. with positive EmA
Alcoholic liver disease	10	6	7
Cryptogenic cirrhosis	8	2	2
Primary biliary cirrhosis	4	0	0
Chronic active hepatitis	2	0	1
Haemochromatosis	1	0	0

Table 6.1: Diagnoses of 25 patients with chronic liver disease tested for IgA EmA

When titres of IgA endomysium antibody were measured, all of the positive samples had strong fluorescence present at 1:10 dilution, and one sample was still positive at 1:20. Five further samples exhibited fluorescence at 1:20, but these were felt to be equivocal results. The remainder were clearly negative at this dilution.

Shortly after these experiments were carried out, the laboratory bought a new fluorescent microscope (Hund H500) to replace our existing Wild M20 microscope. This microscope does not use a dark ground condenser and enables a higher power objective to be used without a reduction in light intensity. This enabled the sections to be examined at higher power and led to the discovery of a different pattern of staining within the cord vessels. The antibodies present in chronic liver disease appear to be directed against nuclear fragments of the smooth muscle cells (picture 6.1) and are therefore not endomysium antibodies.



Picture 6.1: Atypical staining in the IgA endomysium assay seen in chronic liver disease

Summary

From this group of patients it is evident that individuals with chronic liver disease have a higher prevalence of IgA antigliadin antibodies than the normal population. It would appear that chronic liver disease is not associated with false positive IgA endomysium antibodies, and that the earlier findings during the screening of hospital samples were due to the examination of the umbilical cord under low power magnification and our failure to appreciate the difference in patterns of fluorescence. This fluorescence may, however interfere with the interpretation of the microscopy of the section and increasing the dilution of the serum will not necessarily eliminate the fluorescence. It may, in fact, increase the number of false negatives seen in those with coeliac disease. The high total IgA levels would suggest that the high titres of antigliadin antibodies are due to polyclonal expansion of IgA rather than a specific response to gliadin.

CHAPTER 7: RECTAL GLUTEN CHALLENGING USING A RECTAL WASH TECHNIQUE

Introduction

The original ESPGAN criteria for the diagnosis of coeliac disease included the reappearance of pathological changes in the small bowel mucosa in response to reintroduction of gluten in the diet (Meuwisse, 1970). In 1979 these criteria were reviewed, and it was found that gluten challenge was routinely performed by only two thirds of the members of ESPGAN (McNeish et al, 1979). It was also suggested that gluten challenge was not required in all cases as it confirmed the original diagnosis made by the first biopsy in 95% of cases.

Gluten challenging traditionally involves either the addition of 10-15g of gluten powder per day to the food, or allowing the patient to eat 3-4 slices of normal bread per day. A minority of patients develop severe symptoms, but most remain asymptomatic for several weeks or months (McNicholl et al, 1979). A small bowel biopsy is taken when the patient becomes symptomatic, or at three to six months, but even at this point the mucosa may remain normal and there have been reports of the mucosa relapsing only after several years of gluten containing diet (McNicholl et al, 1979; Shmerling and Francx, 1986; Polanco and Larrauri, 1990). Measurements of antigliadin or tissue antibodies have been used to predict mucosal relapse and to time small bowel biopsy (Mäki et al, 1989).

Abnormalities can be found in the rectal mucosa in 5% of patients with coeliac disease (Rubin et al, 1962), and these are thought to be a cell mediated response to gluten antigens present in the faecal stream. Inducing histological changes by exposing the rectal mucosa to gluten has been examined by several groups in the hope that this will provide an alternative to oral gluten challenging in individuals where the diagnosis of coeliac disease is uncertain. These studies have shown rapid histological changes occurring in the rectal mucosa in response to gluten, but these changes are often

subtle, requiring computerised image analysis to detect them. In order for rectal gluten challenging to be used as a routine diagnostic tool in the diagnosis of coeliac disease it should use a readily available, minimally invasive technique. I devised a method of retrieving rectal mucosal secretions using polyethylene glycol and examined whether changes in protein and immunoglobulin secretion could be detected in response to exposure of the mucosa to gluten.

Development of the Technique for Retrieving Rectal Mucosal Secretions

In our laboratory whole gut lavage is a standard technique used for the investigation of blood loss and gut inflammation. This technique is described in detail in Chapter 2, but in brief the patient drinks a large volume of polyethylene glycol plus electrolyte solution (PEG+E) at a steady rate of one litre per hour. Clear fluid passed per rectum is collected and analysed for haemoglobin, albumin, α-1-antitrypsin and total IgG. I felt that if PEG+E could be instilled into the rectum and retrieved after a few minutes, it would collect secretions produced by the rectal mucosa which could then be analysed by our standard laboratory methods.

Initially the technique was used on nine volunteers who were inpatients of the Gastrointestinal Ward. 50mls of PEG+E (Klean-Prep, Norgine, UK) was instilled into the rectum using a rectal catheter. After 15 minutes the fluid was aspirated from the rectum and processed in the same way as WGLF. Fluid was retrieved from three of the nine patients.

In order to improve the technique I attempted to occlude the upper rectum with a balloon in order to prevent the loss of fluid proximally. Initially I attempted to use a urinary catheter with an integral balloon inserted through a disposable sigmoidoscope or proctoscope, but found the catheter was too flexible and could not be advanced easily. I therefore used a 12F Nelaton urinary catheter (Pennine) with a condom attached to the end using surgical suture. This could be advanced through a

disposable proctoscope and inflated with 40 mls of water. Fluid was easily retrieved from a further two volunteers.

The samples from the five volunteers were analysed for haemoglobin, albumin, α-1-antitrypsin and total IgG and the results shown in table 7.1. The assays used for the measurement of these proteins have been specifically designed for use in WGLF which has passed through 4m of gut with a perfusion rate of approximately 20 mls/min. Using the rectal wash technique the fluid is in contact with only 10 cm of gut. In order to calculate a rough estimate of what could be expected in rectal wash fluid one has to assume that the rectum will produce proteins at the same rate per cm as the rest of the gut. The amount of a protein produced by the whole gut per minute can be approximated by multiplying the normal range by the perfusion rate and in rectal wash fluid one would expect to see only one fortieth of the amount produced by the whole gut every minute. The amounts of each protein expected to be seen in 50mls of fluid held in the rectum for 15 minutes are shown in table 7.2.

Patient	Diagnosis	Hb (μg/ml)	Alb (μg/ml)	α-1-AT (μg/ml)	IgG (μg/ml)
KD	Pancreatic carcinoma	1	10	1	6
MC	Weight loss	36	13	4	1
AT	Gastric ulcer, alcoholism	44	312	34	75
EK	Ulcerative colitis	55	212	28	175
MC	Ulcerative colitis	519	472	38	80

Table 7.1: Results of analysis of rectal wash fluid from five patient volunteers for haemoglobin (Hb), albumin (alb), alpha-1-antitrypsin (α-1-AT) and total IgG

Protein	Normal range in WGLF (μg/ml)	Amount expected in 50mls for 15 mins from rectum (μg/ml)
Albumin	0-26	0-4
α-1-antitrypsin	0-19	0-3
IgG	0-10	0-2
Haemoglobin	0-5	0-1

Table 7.2: Expected levels of albumin, α-1-antitrypsin and IgG in rectal wash fluid compared to normal ranges seen in WGLF

In three of the five cases high concentrations of proteins were found, suggesting exudation from the plasma. The haemoglobin was very variable and raised the possibility of bleeding due to trauma. I went on to see if changes could be induced by exposing the rectal mucosa to gluten in susceptible individuals.

Patient Groups

I aimed to examine changes in the rectal wash induced by gluten in patients with classical coeliac disease and compare them to a control group, namely patients with irritable bowel syndrome. I then planned to go on to perform the test on patients with a high likelihood of having low grade pathology coeliac disease such as those with a raised IEL count or those who had commenced a gluten free diet before investigations could be undertaken and had clinically responded. 20 patients attending the Gastroenterology outpatient clinic volunteered to undergo rectal gluten challenge. They were divided into four diagnostic groups which are detailed in table 7.3.

Diagnostic Group	Number of Patients
Coeliac disease	6
Non coeliac	5
Empirical GFD with response	5
Raised IEL count	4

Table 7.3: Number of patients in each diagnostic group undergoing rectal gluten challenge

Rectal Gluten Challenge Test

Aliquots of 100 mls of Klean-Prep were stored at -20°C and a fresh aliquot defrosted prior to each test. Patients were give a bisacodyl suppository to use in the morning before the test to ensure the rectum was empty.

With the patient lying on their left side, the balloon was inserted into the upper rectum with the aid of a disposable proctoscope. The balloon was then filled with 40 mls of water in order to occlude the upper rectum. A rectal catheter was inserted below the balloon and 50mls of PEG+E instilled into the rectum. The rectal catheter was then withdrawn and the fluid left in situ for 15 minutes.

After the allotted time, the rectal catheter was re-inserted and as much fluid withdrawn as possible. The most effective method for withdrawing the fluid was simple drainage since aspiration caused occlusion of the catheter "eyes" with rectal mucosa. Following withdrawal of the fluid, the rectal balloon was also removed and the fluid processed and stored as whole gut lavage fluid. This sample was referred to as the "Baseline rectal wash".

On a second day the patient attended after a further bisacodyl suppository and on this occasion a gluten enema was given. This consisted of 10g crude gluten (Sigma Chemical Co Ltd, UK) in 40 mls of sterile 0.9% saline and was inserted using a rectal catheter. The patient was asked to retain the enema for as long as possible and to re-attend six hours later.

After six hours the rectal wash was repeated and the sample referred to as the "Challenge rectal wash".

Technical Results

The procedure was tolerated well in all patients. With the exception of one woman in the empirical diet group who experienced flushing, abdominal discomfort and urgency of defaecation, no clinical symptoms were induced by the rectal gluten challenge. Two patients were noted to bleed slightly from haemorrhoids following insertion of the rectal catheter.

Fluid was retrieved in all patients, but in varying quantities. The volumes retrieved in each patient are shown in table 7.4. In order to allow for filtration and processing of the sample, a minimum volume of 10 mls was required. If sufficient fluid was not retrieved then the sample was diluted with PEG+E to the minimum volume and this dilution factor was taken into account in all the analyses.

To ensure that the composition of the fluid retrieved was in keeping with PEG+E plus secretions from the rectal mucosa, I measured the concentration of PEG in the samples. A known concentration of PEG was instilled in the rectum in the form of Klean-Prep (59g/l PEG) and so comparing the concentration of PEG in the sample to this value should enable a calculation of how much of the fluid was secreted by the rectal mucosa.

Patient	Baseline vol (mls)	Dilution	Challenge vol (mls)	Dilution
1	15	None	2	1:8
2	15	None	10	None
3	10	None	10	None
4	15	None	20	None
5	10	None	20	None
6	2	1:5	2	1:5
7	1	1:10	12	None
8	5	1:2	1	1:10
9	10	None	2.5	1:4
10	2.5	1:4	10	None
11	2.5	1:4	3	1:3
12	15	None	1	1:10
13	5	1:2	1	1:10
14	15	None	15	None
15	15	None	15	None
16	2.5	1:4	5	1:2
17	5	1:3	15	None
18	30	None	5	1:2
19	2.5	1:4	5	1:2
20	1	1:10	0.5	1:10
21	30	None	4	1:3
22	1	1:10	2	1:5
23	20	None	20	None
24	15	None	20	None

Table 7.4: Volume of rectal wash fluid retrieved from each patient at baseline and challenge and dilution of fluid prior to processing

Measurement of the Concentration of Polyethylene Glycol in the Samples

The concentration of PEG in the rectal wash fluid was determined using the method described in Chapter 2. Filtered/processed fluid was used as more aliquots of this type of sample were available. I tested 18 samples; six paired baseline and challenge samples from the coeliac group, and two paired samples from each of the non-coeliac, increased IEL and empirical diet groups. PEG was used as the standard and a standard curve plotted with the sample values extrapolated from this curve. Klean-Prep was used as a quality control, and contains a concentration of PEG of 59g/l. The dilutional effect of processing the rectal wash fluid was taken into account by multiplying the result by a factor of 1.252. As not all the fluid instilled into the rectum was retrieved it is not possible to calculate the volume of secretions produced by the rectum. One can, however calculate the proportion of PEG in the final sample and therefore the proportion assumed to be produced by the rectum.

The results of this analysis are shown in table 7.5. From the paired data there was no suggestion of the amount of fluid secreted by the rectum in the baseline test differing from that secreted in the challenge test.

The concentration of PEG in samples A and B (marked * and $^{+}$ respectively) are obviously out of keeping with the rest of the samples. In the case of sample A, only a tiny amount of fluid was retrieved (0.5ml) and, as the concentration of PEG was so high, I felt this represented Klean-Prep from within the rectal catheter rather than fluid which had been in contact with the rectal mucosa. Sample B was another small volume sample (1 ml). The concentration of PEG in this sample was extremely low and I am unable to explain this. As the PEG concentration in each of these samples is so different from the rest I felt these were technically unsatisfactory samples and so excluded them from further analysis.

Group	Sample	PEG conc (g/l)	Percentage of sample assumed to
Coeliac	B	56	5%
	C	43	27%
	B	58	2%
	C	38	36%
	B	48	19%
	C	54	8%
	B	43	27%
	C	60*	0
	B	49	17%
	C	2+	97%
	B	34	42%
	C	52	12%
Non-Coeliac	B	58	2%
	C	43	27%
Empirical Diet	B	55	7%
	C	48	19%
Increased IEL	B	46	22%
	C	58	2%

Table 7.5: PEG concentration in 18 rectal wash samples. B=baseline sample, C=challenge sample. Samples A and B are marked by * and + respectively

Measurement of Albumin, Haemoglobin, Alpha-1-Antitrypsin and IgG

The amount of blood in the fluid was established by assaying haemoglobin and the amount of plasma or extracellular fluid exudate by the measurement of three proteins; albumin, α-1-antitrypsin and IgG. These assays were performed by Mr John Bode and Mr Kenneth Humphreys. The results for the coeliac and non coeliac groups are shown in figures 7.1-7.3, with the coeliac disease group divided into treated and untreated patients. It was evident that the amount of haemoglobin in the sample could be dramatically influenced by local trauma caused by the insertion of the rectal catheter and therefore did not reflect inflammatory change within the rectal mucosa.

The levels of the three proteins at baseline did not differ between the two groups, and disappointingly no change was seen after gluten challenge in the coeliac group. Clearly, the aim of my original plan failed and I was unable to use these proteins to detect changes in the rectal secretions of coeliacs in response to gluten. These rectal challenges did, however provide material enabling examination of other potentially valuable parameters of gut immunology such as IgA and IgM, IgA endomysium antibody and proinflammatory cytokines.

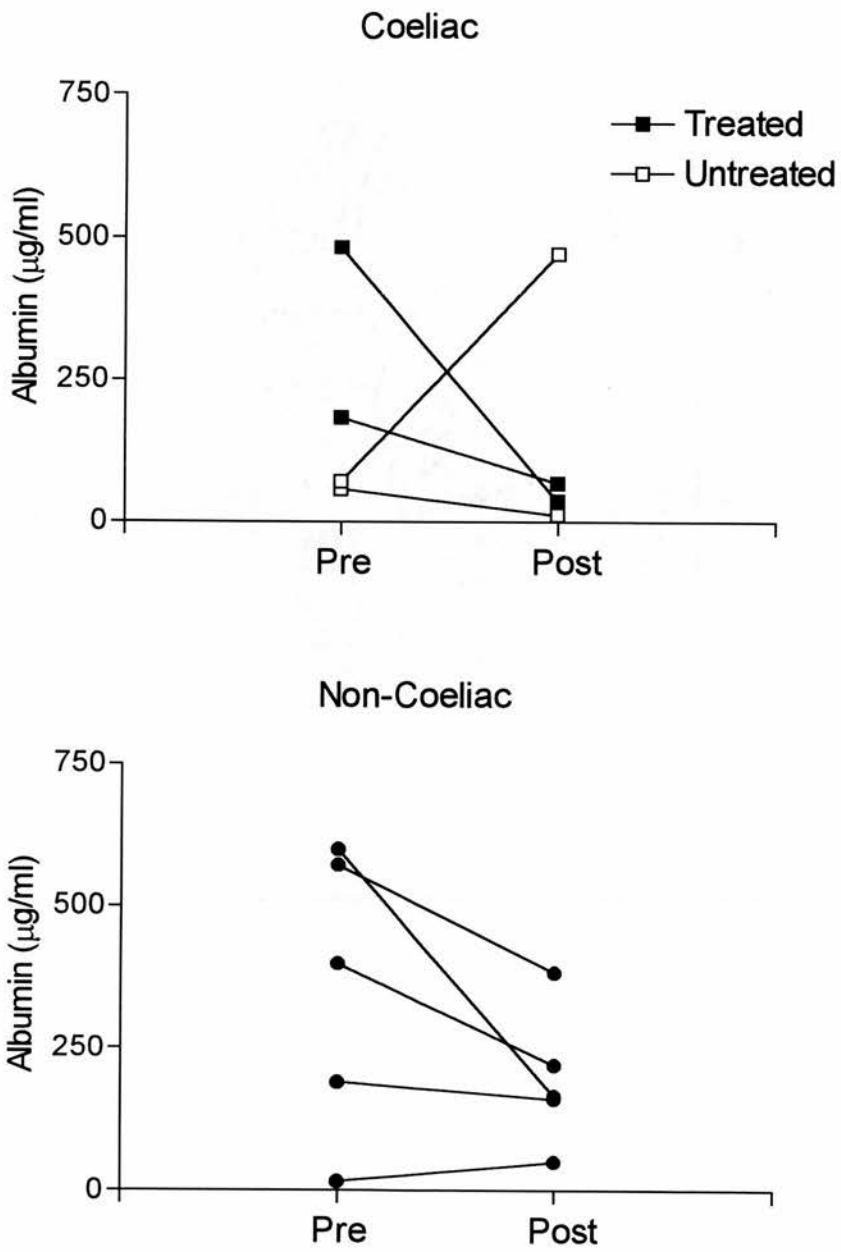


Figure 7.1: Albumin in rectal wash fluid in the coeliac and non-coeliac groups

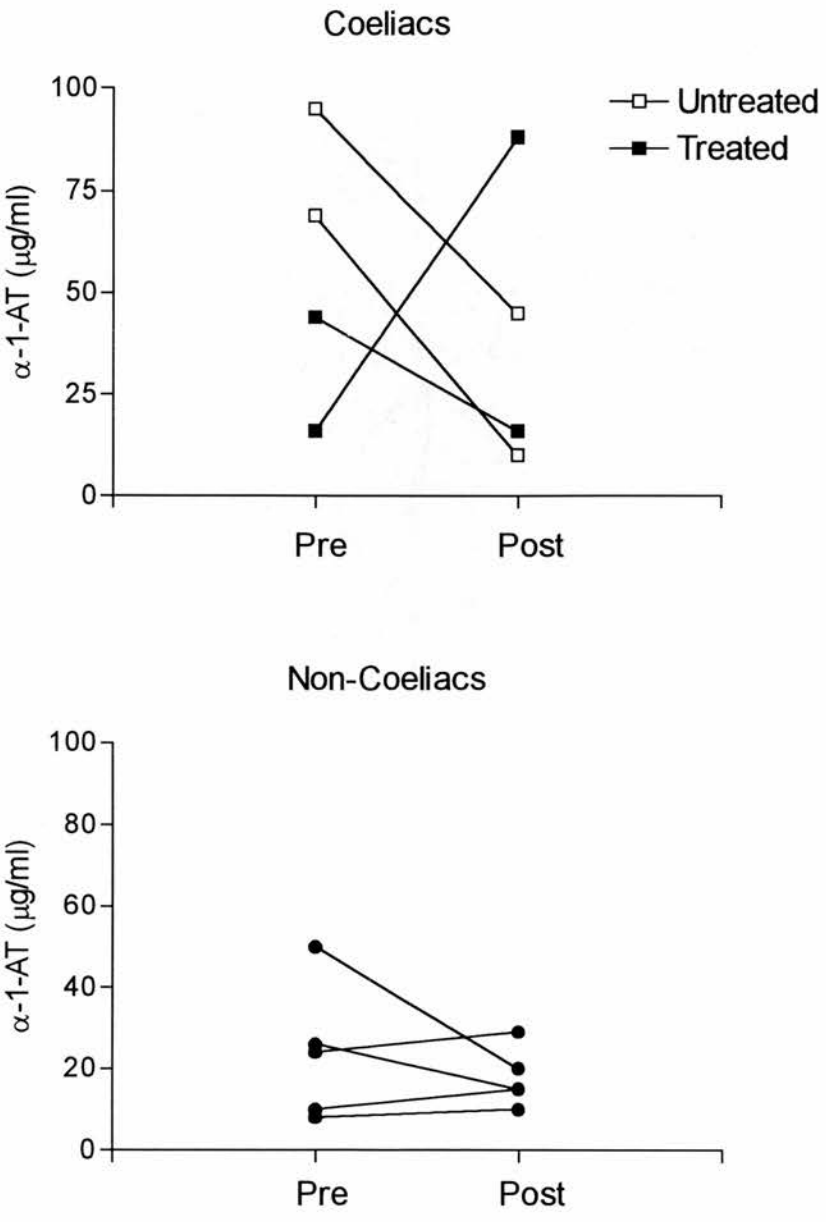


Figure 7.2: α-1-antitrypsin in rectal wash fluid in the coeliac and non-coeliac groups

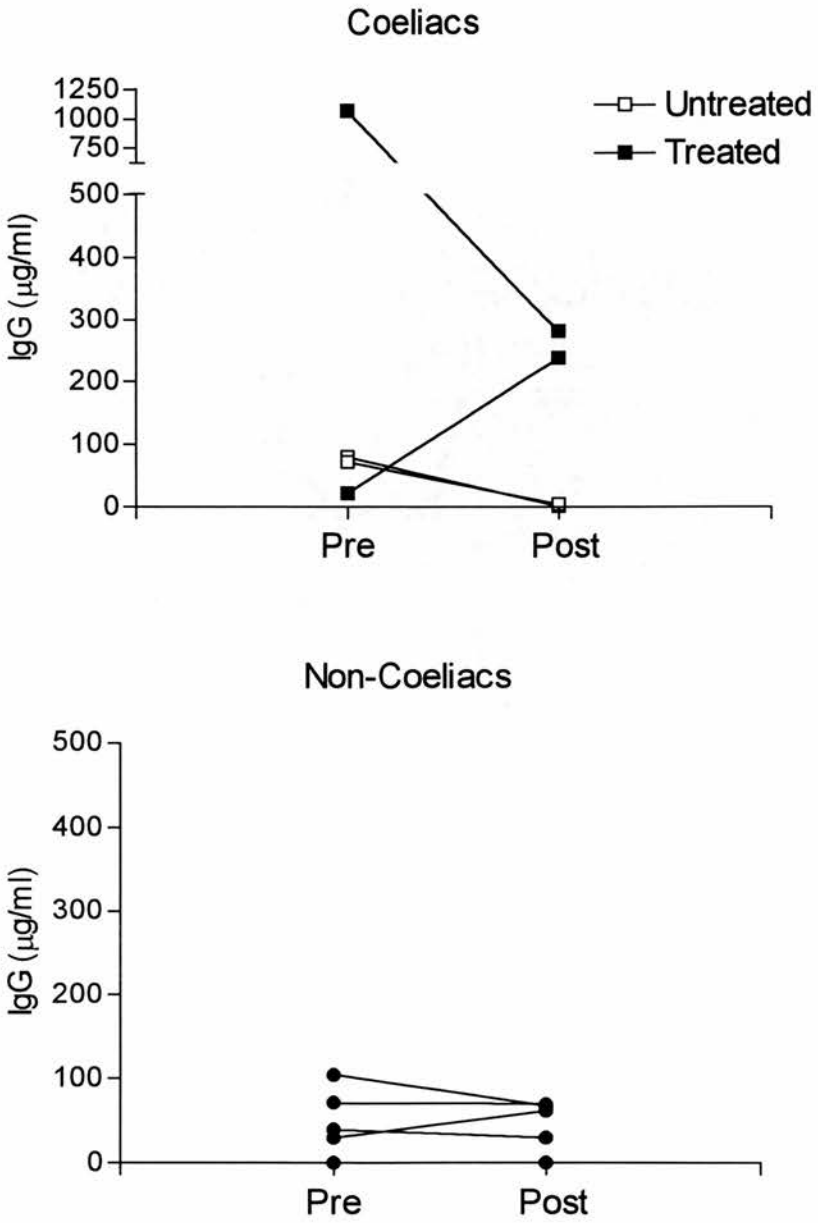
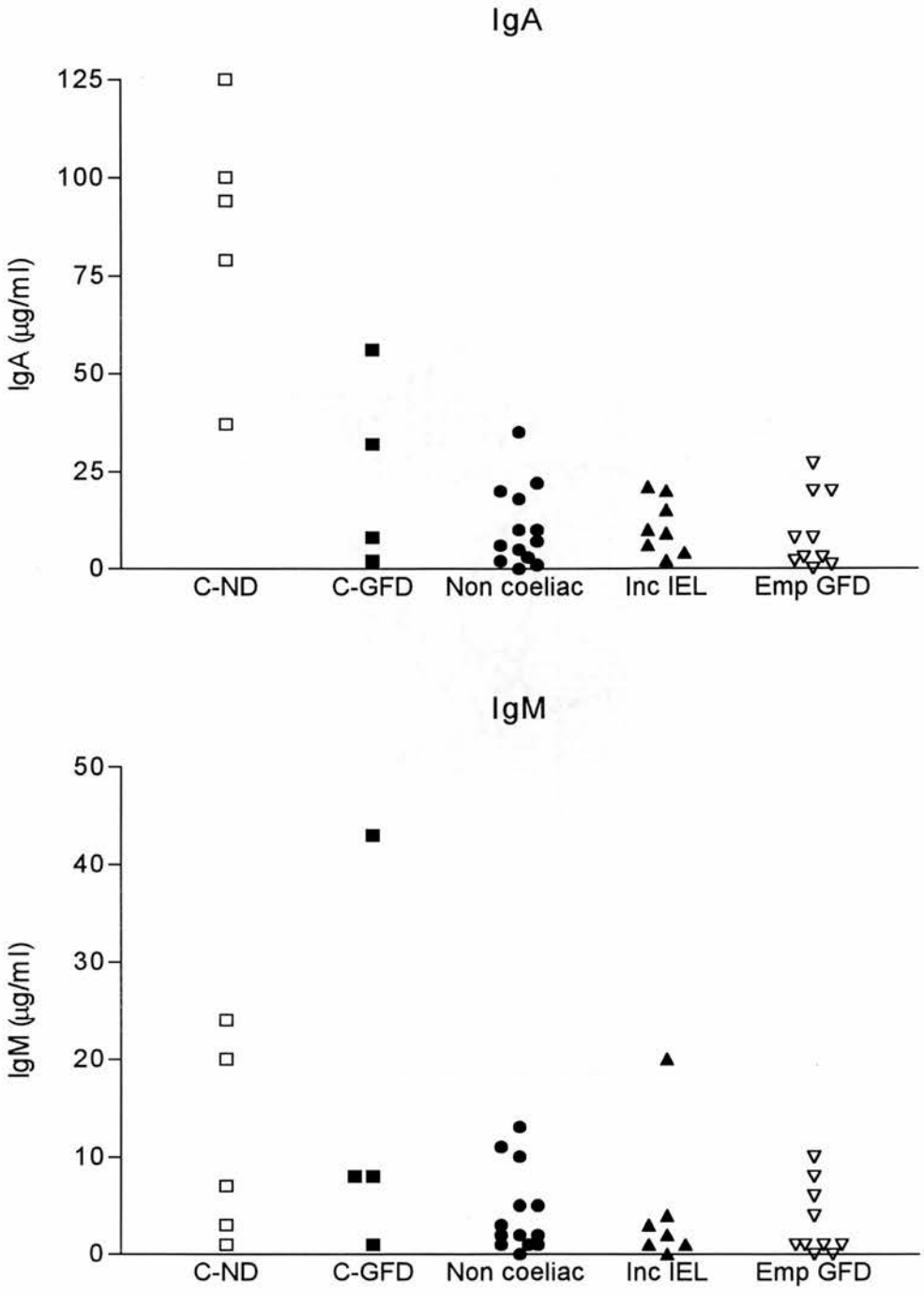


Figure 7.3: IgG in rectal wash fluid in the coeliac and non-coeliac groups

Total IgA and IgM in Rectal Wash Fluid

I went on to measure total IgA and IgM in the fluid using the ELISA method detailed in chapter 2. For the IgA assay, the samples were first diluted to 1:100 before applying to the plate and for the IgM assay, a 1:5 dilution was initially used. Any samples with titres above the highest standard were further diluted accordingly. No aliquots of the fluid were available for three of the samples. As no changes were seen in response to gluten in the three proteins assayed initially the baseline and challenge samples were grouped together for data analysis of the IgA and IgM assays (figures 7.4 and 7.5).

Using the Mann-Whitney Test the IgA and IgM levels in the samples from each group were compared. There were no significant differences between the IgM levels in each group. The IgA in the untreated coeliac group was, however, significantly different to each of the other groups; treated coeliacs ($p=0.037$), non coeliacs ($p=0.002$), the raised IEL group ($p=0.004$) and empirical diet group ($p=0.003$). The levels in the treated coeliac group were not different from those in the other groups. The high levels of total IgA in the five samples from three untreated coeliacs is noteworthy and suggests there is real meaning to the test.



Figures 7.4 and 7.5: Total IgA and IgM in rectal wash fluid in the four patient groups. C-ND = Untreated coeliacs, C-GFD = Treated coeliacs, Inc IEL = Raised IEL group, Emp GFD = Empirical diet group

IgA Endomysium Antibody in Rectal Wash Fluid

As a result of finding IgA endomysium antibody in WGLF from coeliac patients I went on to examine the rectal wash fluid for the antibody. If the antibody is, indeed, formed in response to gluten induced gut damage, I wondered, therefore if it could be found in the baseline rectal wash of coeliac patients in response to reaction of the rectal mucosa to gliadin peptides in the faecal stream. Of even greater importance, I wanted to see if local challenging of the rectal mucosa could stimulate antibody production.

I tested a total of 25 rectal wash samples for IgA endomysium antibody. These were four paired baseline and challenge samples from the coeliac group, two from the non-coeliac group, two from the empirical diet group and one from the increased IEL group. The corresponding pairs for the remaining seven samples had all been used up in previous assays and so were unavailable for this measurement. The assay was performed using the method described for WGLF in chapter 4, ie the fluid was applied to the section of umbilical cord without dilution.

With the exception of one sample, all were negative for IgA EmA. The positive result was found in the challenge sample from a woman with coeliac disease on a gluten free diet. The change from a negative assay on the baseline sample to the presence of IgA EmA in the challenge sample is of great interest, as it suggests this autoantibody is produced in response to local exposure to antigen and by the mucosa in contact with the antigen, rather than by a systemic response. This finding highlights the real need for an ELISA assay to measure endomysium antibody.

Cytokine Measurement in Rectal Wash Fluid

Proinflammatory cytokines are thought to play an important role in the pathogenesis of the mucosal damage seen in untreated coeliac disease. Several studies have shown increased numbers of lamina propria cells secreting interleukin-2 (IL-2), interleukin-6

(IL-6) and Tumour Necrosis Factor-α (TNF-α) in both untreated coeliac disease and a few hours after gluten challenge in treated patients (Przemioslo et al, 1994; Kontakou et al, 1995b; Breese et al, 1994; Jones et al, 1993; Kontakou et al, 1995a). In addition, DQ2 restricted T cell clones have been found to secrete IL-4, IL-5, IL-6 and IL-10 (Nilsen et al, 1995). When planning the study I felt it would be important to see if proinflammatory cytokines were present in the rectal wash fluid of the coeliac patients and how their levels compared to the non-coeliac group. Dr Andrea Lear and Mrs Hazel Drummond performed IL-6 and TNF-α ELISAs on these samples. IL-1 has many functions including the induction of IL-6 secretion and is thought to play an important role in the disease process of inflammatory bowel disease (IBD), as is IL-8 which has a chemotactic effect on both T and B cells. As both IL-1β and IL-8 were being measured in WGLF from patients with IBD and controls, the rectal wash samples were also tested for these cytokines.

The results of cytokine analysis in rectal wash fluid is shown in table 7.6. IL-1β and IL-8 were each measured in 44 of the 45 samples. There was insufficient fluid for both assays in two samples. IL-6 was measured in 32 of the 45 samples, again due to lack of fluid in 13 samples. TNF-α was measured in a total of 17 samples and was detectable in only one of these, a challenge sample from a patient in the empirical diet group. In view of the shortage of sample material and the failure to detect this cytokine in the vast majority of samples tested, no further analysis of TNF-α was performed in the fluid. Wherever possible the cytokine analysis was repeated at differing dilutions in order to achieve an absolute concentration, but if sample material was in short supply, the value was expressed as greater than the top standard.

There was no difference in IL-1β measured in baseline and challenge samples in either group. The levels in each group were therefore compared (figure 7.6). There was no significant difference between the untreated coeliacs and any other group, but the treated group were significantly different from the three colitics ($p=0.036$).

Group	Sample	IL-1β (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)
Colitis	B	>600	No sample	>1000
	B	180	No sample	>1000
	B	102	90	>1000
Emp Diet	B	6	70	6
	C	37	No sample	No sample
	B	7	6	234
	C	164	74	>1000
	B	600	57	>1000
	C	36	22	898
	B	2	0	38
	C	0	0	14
	B	68	92	2593
	C	17	10	2406
Inc IEL	B	15	No sample	95
	C	42	No sample	608
	B	234	19	167
	C	21	92	0
	B	No sample	19	307
	C	298	574	6314
	B	13	21	316
	C	8	0	211
Non Coeliac	B	0	No sample	20
	B	0	0	799
	C	265	9	>1000
	B	22	No sample	497
	B	335	162	>1000
	C	111	38	>500
	B	0	0	16
	C	0	0	13
	B	40	No sample	1347
	C	15	No sample	289
	B	5	No sample	859
	B	6	0	267
	C	4	11	109
Coeliac (GFD)	B	16	4	83
	C	19	3	384
	B	14	0	57
	C	14	0	49
Coeliac (ND)	B	18	13	>1000
	C	7	No sample	65
	B	108	101	5966
	B	411	>500	>1000
	C	750	456	>1000
	B	555	No sample	>1000
	C	7	No sample	68

Table 7.6: Cytokine analysis of rectal wash fluid

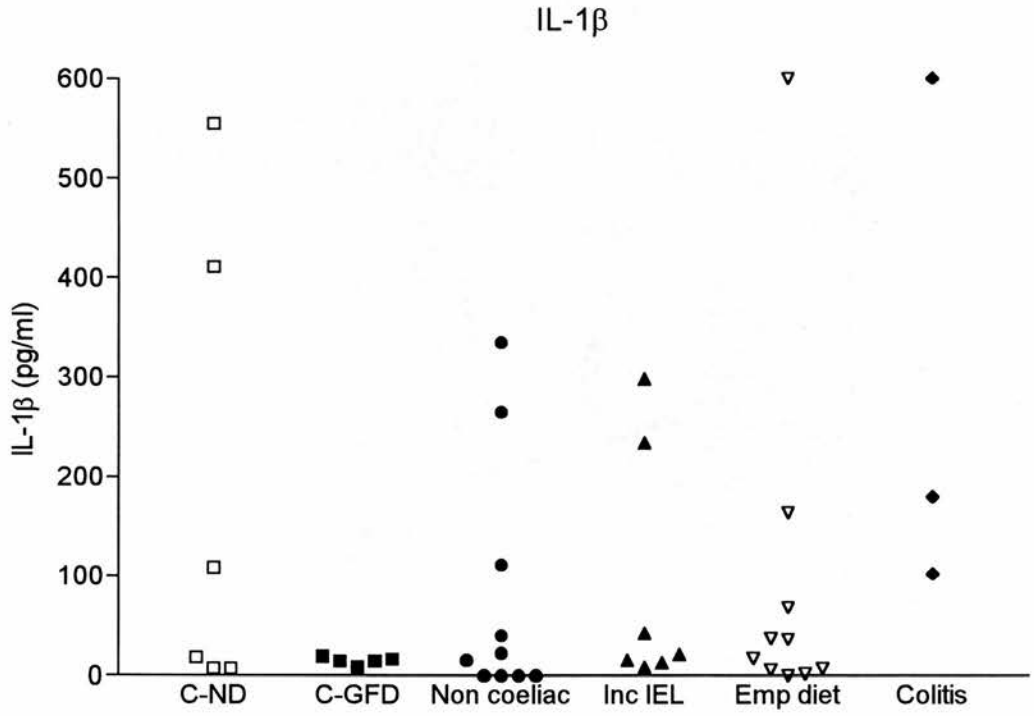


Figure 7.6: Levels of IL-1β present in rectal wash fluid in different diagnostic groups. The treated coeliac group (C-GFD) was significantly different from the colitis group (p=0.036)

One of the functions of IL-6 is to stimulate secretion of IgA by plasma cells. In view of the interesting results in the IgA assay, I compared these results to the levels of IL-6 in the same samples (figure 7.7). There was, however no correlation between the two parameters in these samples.

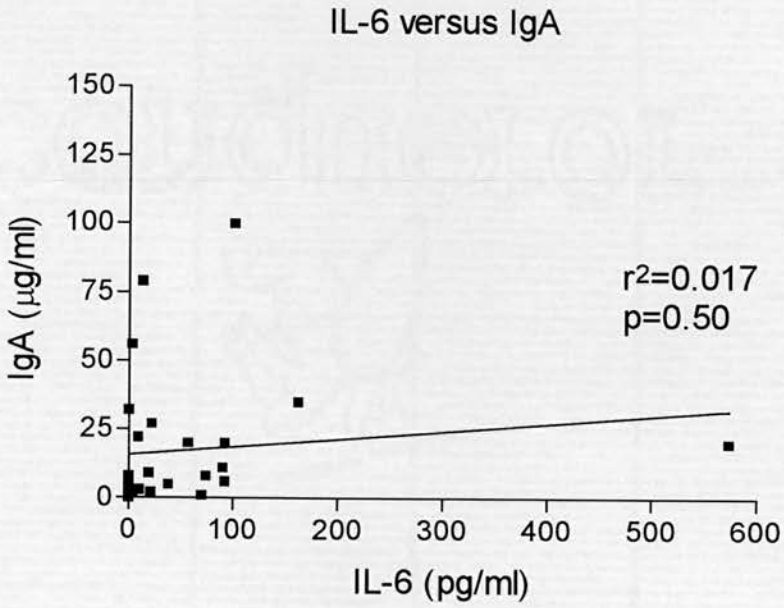


Figure 7.7: Comparison of IL-6 and IgA in 28 rectal wash samples

Wide variation can be seen in the IL-8 levels in rectal wash fluid in all the groups. Although the paired baseline and challenge data looked promising, when analysed no differences were found between the groups, with the exception of the baseline samples in the untreated coeliac and the possible coeliac group (made up of the combined raised IEL and empirical diet groups) ($p<0.05$). This is very likely to be due to the extremely small number of samples in the untreated group. The paired pre- and post-challenge IL-8 levels are displayed in figure 7.8.

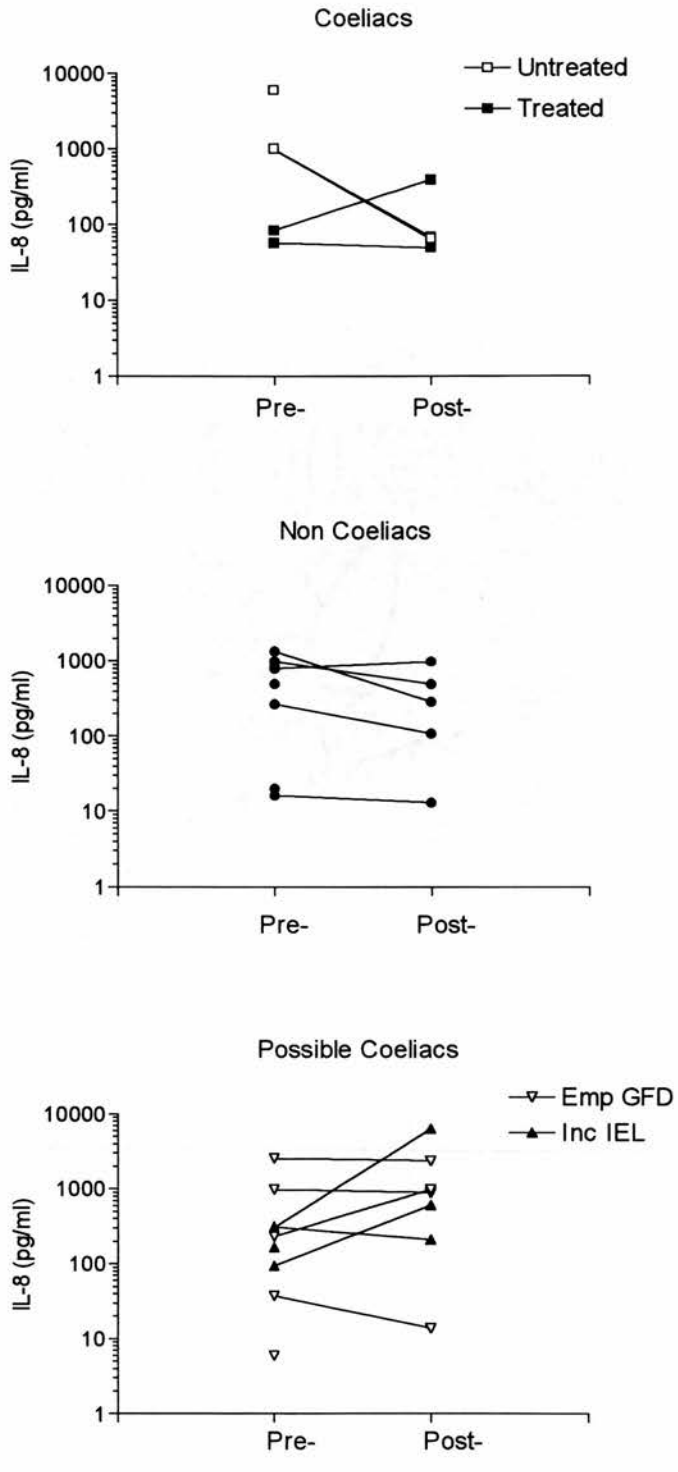


Figure 7.8: Pre- and post-challenge levels of IL-8 in rectal wash fluid from each diagnostic group

Discussion

Gluten challenging is required to confirm the diagnosis of coeliac disease in a minority of patients. Oral gluten challenging, however, is often time-consuming and may induce distressing clinical symptoms in some patients. Other patients do not develop symptoms, leaving a dilemma as to when to perform small bowel biopsy. The finding of a histologically normal small bowel also produces confusion as to whether this truly reflects a negative gluten challenge, or simply that relapse has not yet occurred. For these reasons, an alternative method for gluten challenging is needed and the positive results from other centres suggests rectal gluten challenge may be the way forward. In order for this to take place, the technique employed must be simple to perform as well as reliable in its results. I attempted to use a technique of rectal washing, avoiding the need for multiple rectal biopsies and expert histological analysis. Although the tests performed on the fluid were multiple and appear complex, each individual assay is simple to perform and could be adopted by most motivated laboratories. Unfortunately, I was unable to demonstrate any clear difference between the coeliac and non-coeliac groups in any parameter measured and the responses to gluten were wide and varied with no significant difference between the groups. This may be due to the small size of each group, which reflected the difficulty I experienced in recruiting volunteers for this study. Many of the patients who did volunteer did so because their diagnosis was not clear-cut, and so would be the patients in whom a reliable and accurate rectal gluten challenge would be the most useful. Many had commenced a low-gluten or gluten free diet at home before attending the outpatient clinic and had experienced a clinical response. Their investigations were either normal or equivocal reflecting either an effect of their diet or an alternative diagnosis to coeliac disease. The patients in the increased IEL group could not be definitively diagnosed as gluten sensitive and so could not be included in the coeliac group.

This work did produce interesting results aside from the effect of local instillation of gluten. Although it is impossible to directly compare the levels of proteins found in rectal wash fluid to WGLF as the samples are collected in different ways and are

designed to measure different sections of the gut, I was surprised by the often extremely high levels of proteins found. I initially thought that the high levels seen in the patients with active proctitis must reflect acute inflammation, but was surprised to see similar levels in individuals with normal rectal mucosa. As I have already mentioned, WGLF has passed through the whole gut prior to collection whereas the rectal wash fluid was in contact with approximately 10 cm of mucosa before retrieval. One possible explanation would be a difference in perfusion rates, but this did not seem to be the cause as the levels were often higher than the predicted ranges for the proteins which were calculated, albeit with a great deal of assumption. Another explanation would be an artificially low recovery of protein in WGLF due to digestion or degradation in the gut during the test. This is known to be the case for albumin but not for α-1-antitrypsin or IgG.

Another surprising finding was the variable volume of fluid retrieved. In no patient was the initial volume of 50mls retrieved. In the beginning, I felt this was due to proximal loss and this led to the development of the proximal balloon technique. Despite this, however, in many patients only extremely small volumes could be recovered. In many, fluid did not immediately flow from the rectal catheter on reinsertion and this could be rectified by manipulating the tip of the catheter anterolaterally. In some patients, however, no manoeuvre resulted in increased volumes being retrieved. As none of the patients experienced incontinence during the test, the fluid must have been lost proximally despite the placement of the balloon. Other centres have used commercially manufactured silastic multiple lumen tubes to perform perfusion studies in patients with inflammatory bowel disease and I attempted to purchase one of these to see if the technique could be improved, but unfortunately these are custom-made at very high cost and so were unavailable to me. In the future, however, this would be worth investigating to see if this technique can be taken further as a minimally invasive method of investigating rectal inflammation.

The results of the cytokine assays were very interesting. High levels of IL-8 were seen in many of the patients, including the non-coeliac group. Again this may reflect the

difference in perfusion between the two techniques. Detecting IL-6 at all was a great surprise when it had been completely undetectable in WGLF and this led to a set of interesting experiments revealing the immediate disappearance of IL-6 in filtered WGLF. We felt this may reflect the binding of the cytokine to soluble receptors present in the fluid.

Although it was a great disappointment to me that this technique did not provide a method for performing rectal gluten challenging, I feel overall that the work has had positive results of interest both to myself and to colleagues within our Unit.

CHAPTER 8: GENERAL DISCUSSION

Gluten sensitive enteropathy is a condition comprising a broad spectrum of both mucosal pathology and clinical symptoms. In this thesis I have presented work relating to three aspects of the condition with the aim of augmenting the diagnostic process in patients with minimal changes in small bowel biopsy pathology or in whom the diagnosis of coeliac disease is not clear-cut.

In coeliac disease, an infiltration of small intestinal intraepithelial lymphocytes occurs and, increasingly, individuals are being identified with normal mucosal architecture but raised IEL counts. Many of these patients have dramatic symptoms which respond to exclusion of gluten from the diet. I have presented work describing the development of a technique to quantify IEL according to the epithelial surface length using computerised image analysis to replace our traditional technique of counts of epithelial cells and IEL by light microscopy. The computerised technique has been validated by comparison of counts performed on a group of small intestinal biopsies using both methods, counts obtained by two independent observers from the same sections, counts in multiple biopsies taken on the same occasion, and by counting IEL in two sections taken 100μm apart from a single large jejunal biopsy. In all of these experiments, my computerised technique has been shown to be both reliable and reproducible and, on the basis of this work, has now been adopted by our laboratory as the routine method for performing IEL counts on patient samples.

I used the technique to perform counts on 250 small bowel biopsies with normal mucosal architecture. I found IEL counts of over 50 cells/mm in 30 of these, seven from known treated coeliacs and the remainder from individuals with various clinical symptoms in whom coeliac disease was considered to be a possible underlying cause. On the basis of these raised IEL counts, the possibility of low grade pathology coeliac disease was discussed with the patients and nine of the 23 agreed to exclude dietary gluten for a trial period. A clinical response was seen in all but one supporting this

diagnosis. These results are in keeping with similar findings from my predecessor, Dr Eduardo Arranz.

Although counts of total, $CD3^+$ and $\gamma\delta^+$ IEL are extremely useful in the diagnosis of both classical and low grade pathology coeliac disease, we are still unclear as to the function of these cells and their role in the development of enteropathy. They do demonstrate some cytotoxic abilities, especially against targets coated with enterotoxins, and they produce cytokines possibly as protection against viral or bacterial infection. It is also thought that they play a role in the development of oral tolerance to ingested antigens, and this mechanism may be abnormal in coeliac disease. IEL infiltration may therefore be a local T-cell-dependent response to an ingested antigen, and indeed the counts of total and $CD3^+$ IEL do fall on withdrawal of dietary gluten in gluten sensitive individuals. Despite this, however, the IEL infiltration, per se, does not seem to damage the mucosa as demonstrated by the occurrence of infiltration in otherwise normal biopsies. Another dilemma is the apparent failure of $\gamma\delta^+$ IEL to return to normal levels despite treatment with a gluten free diet for several years. It may be that counts fall very gradually and reduction to normal levels takes many years.

Over the next few years, work in this field should be aimed at the identification of the role played by these cells in the development of gluten sensitive enteropathy and also at the ability of coeliac individuals to develop oral tolerance to dietary antigens, which can be achieved by using a novel protein such as keyhole limpet haemocyanin. If these aspects of the disease are understood more fully, it may be possible to develop alternative treatment strategies for those in whom lifelong dietary exclusion is not sufficient to induce remission of the enteropathy, and for patients who are unable to comply with diet treatment recommendations.

In the course of my work I, along with Mr Bode, have established the IgA endomysium antibody assay using indirect immunofluorescence with human umbilical cord as the immunogenic substrate. Since the description of this technique by Ladinser et al in

1994 it has been rapidly adopted throughout the world making the measurement of this sensitive and specific coeliac related antibody widely available. We have validated our method by initially testing stored serum samples from coeliac and non-coeliac patients and then by prospectively testing serum submitted to the laboratory for analysis of IgA antigliadin antibody. Our assay shows a sensitivity of 100% and specificity of 94.6% and is now established in the laboratory's NHS analytical service.

In the light of the positive response of eight patients with raised IEL counts to gluten free diet, I measured IgA EmA in stored serum samples from 20 of the 23 with raised counts. Three of the eight responders did demonstrate the antibody in their serum, and none of the patients with self-limiting illness had positive tests. Testing the antibody in these patients is therefore useful as, although a negative result does not exclude a response to gluten withdrawal, a positive result suggests that the biopsy findings do reflect low grade pathology gluten sensitivity and a clinical improvement will be seen following the commencement of a gluten free diet.

The positive predictive value of IgA endomysium antibody for coeliac disease has led to its use in the identification of undiagnosed cases in high risk populations, such as individuals with type I diabetes or Down's Syndrome and in first degree relatives of coeliacs. We have looked at a large number of insulin-dependent diabetics; more than 600 adults and over 130 children. We found 18 patients with positive IgA EmA and offered each of them the chance to discuss the implications of this result. I was surprised to find that of the 17 who attended the outpatient clinic, 70% had symptoms which could be attributed to coeliac disease. 70% also had haematological or biochemical abnormalities, the commonest being hypomagnesaemia, which was seen in ten of the patients. At the time of submission of this thesis only four patients have had small bowel biopsies taken. All of these patients had severe symptoms, predominantly diarrhoea, and I have been surprised to see, therefore, that only one patient had classical villous atrophy and crypt hyperplasia and two patients had normal IEL counts. It may be that the immune response in diabetes is different to that of non-diabetic individuals and that mucosal damage is mediated predominantly by complement activation. Alternatively, the mucosal lesion may be very patchy and the

duodenal biopsies may have been taken from areas proximal to affected gut. These four patients have been offered treatment with a gluten free diet with subsequent improvement in their symptoms.

As a result of the positive findings of this study I feel that all patients with type I diabetes should be offered serological screening for coeliac disease using IgA EmA and antigliadin antibodies.

In order to assess the prevalence of coeliac disease in a hospital population rather than in healthy volunteers or blood donors, we tested for IgA EmA and AGA in 300 plasma samples submitted for full blood count assessment and 600 stored serum samples from patients attending our own unit with gastrointestinal symptoms. We found 27 samples positive for both antibodies; eight were known to have coeliac disease, one had dermatitis herpetiformis, and five had alcoholic liver disease. Two patients agreed to undergo endoscopic biopsy, of which one confirmed coeliac disease and the other is still outstanding.

Although this study only revealed one new case of coeliac disease it led to the investigation of chronic liver disease as a cause of a false positive result in the endomysium antibody assay. This work revealed an atypical fluorescent staining pattern produced against human umbilical cord by serum from individuals with cirrhosis. I also found high levels of total IgA in these patients and high titres of IgA AGA.

In the future I think that screening for coeliac disease will become more common, especially in the diabetic population and because of this I took earlier work by my colleagues further by developing a method for measuring IgA EmA in material eluted from dried blood spots. This work looks extremely promising, as does my predecessors' work with antigliadin antibodies from eluted spots. The assays obviously require more work on their development and validation but I am sure they will prove to be valuable tools in screening for coeliac disease.

As selective IgA deficiency is associated with gluten sensitive enteropathy, relying on IgA antibodies to detect coeliac disease will lead to missed diagnoses and delayed treatment. In view of this, I modified the IgA EmA assay to detect the IgG isotype of the antibody and initially tested a woman with dysimmunoglobulinaemia and jejunal subtotal villous atrophy which was refractory to gluten free diet. The IgG antibody was positive and became negative following her clinical response to immunosuppressive treatment with cyclosporin. I have since found IgG EmA in serum from seven further IgA deficient patients, all of whom have now been diagnosed with coeliac disease.

In order to assess the prevalence of IgG EmA in patients with coeliac disease and normal total IgA levels I tested 38 IgA EmA positive serum samples for the IgG isotype and found both classes of antibody present in 18%. This figure is in keeping with results from another group.

The modification of the IgA assay has, therefore, proved to be a useful tool for detecting coeliac disease in IgA deficient individuals. We have only been able to test a small number of patients as we have used only diagnostic samples sent in to our routine analytical service. To my knowledge, there have been no large studies to assess the prevalence of IgG EmA in IgA deficient coeliacs and I think this would be extremely valuable to perform, but it will require collaboration between several centres in order to increase the number of samples available.

The site of production of endomysium antibodies and the nature of the antigen against which they are directed are, as yet, unknown. The finding of tissue antibodies in intestinal secretions, and more recently in whole gut lavage fluid, may reflect either mucosal production or spill-over from a systemic origin. I examined stored WGLF and serum from a group of coeliacs and found IgA EmA in the fluid in three patients, one of whom was on a gluten free diet and did not demonstrate the antibody in serum. This would therefore suggest that the antibody is produced by the mucosal plasma cells and at high titres spills over into the systemic circulation.

The function of this antibody is unknown at the present time. It may represent an autoantibody which causes the mucosal lesion seen in coeliac disease but, alternatively it may be produced secondarily after T-cell activation has induced mucosal damage. Obviously work in the future will be directed towards the identification of the antigen which will enable the development of quantitative assays in the form of ELISAs. Having an accurate objective measurement of antibody titres will help the understanding of the relationship between this antibody and the natural history of the disease. Our laboratory's expertise in the technique of whole gut lavage offers an ideal opportunity to further examine this material for endomysium antibody in order to establish its function and source.

Other groups have investigated changes in rectal mucosal histology in response to local exposure to gluten and have suggested that this method of gluten challenging could, in the future, replace oral gluten challenging. The changes induced are very rapid, the majority being maximal at 6 hours, and the results appear reproducible. The histological changes are subtle, however, and require expertise and computerised image analysis to detect them. The interpretation of changes is also difficult and this may account for the majority of studies being carried out in only one or two centres.

I investigated the possibility of analysing changes induced in the rectal mucosa by local gluten exposure using a rectal wash technique. I felt this would be less invasive than performing multiple rectal biopsies on several occasions. I devised a technique for instilling PEG+E into the rectum and then retrieving the fluid after a period of time. The fluid was then processed and analysed in the same way as WGLF. I hoped that, if changes in mucosal permeability could be induced in sensitive individuals, increased exudation of proteins such as albumin, α-1-antitrypsin, and immunoglobulins could be detected in the fluid.

Unfortunately this theory was not borne out by the results. There was a great deal of variability in the concentration of PEG in the fluid retrieved from different patients, suggesting different amounts of fluid secreted by the rectal mucosa. There was no

consistency within groups and so I could not say that one group was more likely to produce higher volumes of rectal secretions in response to gluten challenge than another. I also found that the volume of fluid retrieved after the test varied from patient to patient and also between baseline and challenge tests in the same patient. In no patient was the instilled volume of 50mls retrieved. Occlusion of the upper rectum did not improve this and I presume that proximal leakage of the fluid occurred despite the placement of an inflated balloon. There was no significant difference in the albumin, α-1-antitrypsin or IgG at baseline between the groups and, more importantly, no significant difference following challenge either.

I went on to examine other parameters in the fluid including total IgA and IgM, and found that levels in the fluid from untreated coeliacs was significantly higher than those in treated coeliacs, non coeliacs, individuals with increased IEL and those treated empirically with gluten free diet. There were no differences in IgM levels between any of the groups.

In the light of the finding of IgA endomysium antibody in WGLF from coeliacs, I examined the rectal wash fluid for this antibody. Out of a total of 25 samples IgA EmA was found in only one, a post-challenge sample from a treated coeliac. Again, this supports the concept of mucosal production of the antibody.

We went on to examine the fluid for pro-inflammatory cytokines and I was surprised by the number of samples found to contain high levels of IL-1β, IL-6 and IL-8. Again, no significant difference was found between the levels detected in each group. One of the functions of IL-6 is to stimulate production of IgA by plasma cells, but there was no correlation between the IL-6 levels and total IgA in the fluid.

For this work I instilled gluten into the rectum and performed the rectal wash technique six hours later. This period of time was chosen on the basis of reports of maximal histological changes occurring at six hours post-challenge. It may be that this period of time is not long enough to induce exudation of proteins from the plasma and

performing the rectal wash after, say 24 hours may produce different results. This may also be the case for IgA EmA and a longer challenge may produce positive assays in more of the samples.

My technique for performing the rectal wash was not ideal and I would probably have had greater success if I had used a custom-made catheter. If this work is continued, then a manufactured all-in-one double balloon perfusion catheter should be used. The time period between the gluten challenge and the test should also be adjusted to see if changes can be induced in gluten sensitive individuals. If changes can be induced, the test should also be performed after challenge with another dietary antigen such as β-lactoglobulin or ovalbumin in order to show that the changes are specific to gluten. Although rectal gluten challenging has offered promise as an adjunct to the diagnosis of coeliac disease, with the advent of the endomysium antibody assay we may find that it is never developed for clinical use but will remain as a research based method.

Over the next few years I am sure we will see dramatic developments in the understanding of the pathogenesis of coeliac disease and also in the diagnostic criteria used for the condition. Already, jejunal biopsy by peroral capsule is being superseded by endoscopic distal duodenal biopsy using large cupped, spiked biopsy forceps. This technique is safe, rapid and can be performed using either local anaesthetic throat spray or sedation, making it acceptable to the patient. Using the specifically designed forceps enables the collection of large, multiple biopsies which are perfectly adequate for a histological diagnosis to be made.

The advent of a widely available assay for measuring IgA EmA has already changed our approach to the investigation of patients with diarrhoea, weight loss or anaemia. Previously, patients with such symptoms would have had a series of invasive investigations including small bowel biopsy, performed as a matter of routine, but now serum antigliadin and endomysium antibodies are checked as first line investigations before biopsy is embarked upon.

The accuracy of endomysium assays, throughout the world, is now so good that the need for performing small bowel biopsies in symptomatic, antibody positive individuals is being questioned. In my own work in the diabetic patients, three of the biopsies have been either entirely normal or very mildly abnormal, but despite this the patients' symptoms have improved on a gluten free diet. In these patients, therefore, the decision to treat the patients has been based on the endomysium antibody result anyway, with little being added by the biopsy.

Another change to the diagnostic criteria will be the inclusion of the presence of HLA-DQ2. I am sure we will find in a few years that individuals will be diagnosed as gluten sensitive on the basis of symptoms, a positive endomysium antibody, their HLA type and their clinical response to a gluten free diet.

Over the next few years I hope that work in coeliac disease in Edinburgh will continue in the following areas:

- Assessment of oral tolerance to novel dietary antigens in coeliacs compared to normals
- Assessment of the prevalence of IgG EmA in IgA deficient coeliacs
- Measurement of IgA EmA in WGLF from treated and non treated individuals
- The development of a quantitative ELISA for IgA EmA when the endomysium antigen is identified
- Further assessment of cytokine levels in both rectal wash fluid and WGLF from coeliacs compared to non coeliacs and those with inflammatory bowel disease

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APPENDIX

ALBUMIN IN WHOLE GUT LAVAGE FLUID

PRINCIPLE OF METHOD

When human albumin to be assayed reacts with its specific antibody, precipitating immunocomplexes are quickly formed in the presence of polyethylene glycol. If the antibody is present in large excess, these precipitates produce a turbidity which is related to the concentration of albumin in the sample. The turbidity is photometrically measured at the wavelength 340nm. Absorbance readings obtained by assaying calibration standards are used to generate a standard curve, from which the concentration of albumin in the sample is derived.

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HAZARDOUS SUBSTANCES AND NATURE OF HAZARD

CHEMICAL

sodium azide: toxic - see COSSH form no 107
phenyl methyl sulphonyl fluoride: toxic - see COSSH form no 104.

BIOLOGICAL

Whole gut lavage fluid (WGLF) is potentially infectious and should be handled appropriately. Procedures for cleaning glassware and disposal of tips which have come in contact with these materials should be strictly adhered to.

PRECAUTIONS

Safety measures as detailed in the Departmental safety handbook must be followed. Appropriate COSHH forms should be read and understood before undertaking this assay.

SPECIMEN REQUIREMENTS

WGLF: 6 x 1.5 ml samples of whole gut lavage fluid (filtered and processed) should be aliquoted into 2 ml microtubes with screw lids (Sarstedt, UK). Samples should be stored at -70 C. The procedure for filtering and processing lavage fluid is described below.

1. 2 x 25 ml samples of clear WGLF are collected from the patient and processed withion 15 minutes where possible. If the collected samples do not appear very clear or do not filter relatively quickly then centrifuge the samples at 3600 rpm for 5 minutes.

2. Filter 10 - 15 ml into a universal container, using Whatman GF/A 12.5 cm filter paper. Transfer 10 ml to another universal container.

3. Add 1 ml SBTI. Mix.
Add 0.56 ml NaEDTA. Mix.
Add 0.24 ml PMSF . Mix.
Add 0.12 ml sodium azide. Mix.

Leave for 2 minutes.

Add 0.6 ml new born calf serum. Mix, and dispense aliquots.

SAMPLE STORAGE AND STABILITY

samples of lavage should be kept stored at -70. These samples are stable for up to 1 year at least.

INSTRUMENTATION

Turbidity is measured using the PU8640 spectrophotometer (Room 1).
Switch on main power. Side of instrument.
Set to ABS mode
Set wavelength to 340 nm
Set delay to 0.01
Set volume to 0.8
Set Temp Off
Store as program 4

Lavage samples are diluted with PEG reagent using the GILSON DILUGIL V. This is set to sample 0.05 ml and to dispense 0.95ml.

Assays are carried out using 2 ml polystyrene tubes (LIP Ltd) 64 x 11 mm; code no 30908.

REAGENTS

All reagents are analar grade and obtained from Merck (BDH) unless otherwise stated. These are stored in the fridge in room 1 unless otherwise stated.

FOR PROCESSING WGLF

1. Phosphate Buffered Saline: (PBS - Sigma) 1 tablet dissolved in 200 ml distilled water to give pH 7.2.

2. Soy Bean Trypsin Inhibitor: (SBTI - Sigma):Dissolve 0.1g per 100 ml PBS. Solid kept at -20 C.

3. Disodium EDTA: Add 11 g to 100 ml distilled water. Dissolve to form a saturated solution. Store at room temperature. Solid stored in room 1 chemical cupboard.

4.Phenylmethylsulphonylfluoride: (PMSF - Sigma) 0.1M in 95% alcohol (IMS). Store solid in room 1 chemical cupboard.

5. Sodium Azide: 2g/100 ml distilled water.

FOR WGLF ALBUMIN ASSAY

1. PEG Reagent: 40g polyethylene glycol
6000 (biochemical grade), 6g tris, 2g Tween 20 (Sigma), and 1g sodium azide dissolved in 800 ml distilled water, adjusted to pH 7.0 with dilute hydrochloric acid and made up to 1 litre volume with distilled water.

2. Anti Human Albumin (sheep): (SAPU - code S034-205). Store frozen in aliquots of 0.7 ml at -20 C.

3. Antibody Reagent: Dilute sheep anti human albumin serum 1 in 40 with the PEG reagent on the day of assay.

4. Diluent: 9g sodium chloride, 60g PEG 3350 and 1g sodium azide in 1 litre solution distilled water.

CONTROL MATERIAL

STANDARD

1. Stndard serum SPS01: (Protein Reference Unit - Sheffield). Keep at 4 C.

2. Albumin: Dilute standard serum with diluent (Reagent 4) to give the following range of standards : 0,10,20,50,100,200 ug/ml. Prepare fresh on day of assay.

eg for a standard with albumin concentration 40.5 g/l and a1-antitrypsin 1.52 g/l. Take 100 ul standard and dilute it with 0.66 ml of diluent, to give an a1-antitrypsin 200 ug/ml standard.
Dilute 0.2, 0.1, 0.05, and 0.05ml of this with respectively 0.2, 0.3, 0.45 and 0.95 ml diluent to give further standards of 100, 50, 20, and 10 ug/ml. To get a series of albumin standards with similar concentration dilute 37.5 ul of each of the a1-antitrypsin standards with 1.0 ml diluent.

QUALITY CONTROL MATERIAL

This consists of a lavage sample from a normal volunteer (blood donor) to which serum from the same volunteer is added to simulate leakage as in inflammatory bowel disease.
Store in aliquots of 0.5 ml at -70 C. Stable for up to 1 year at this temperature.

ANALYTICAL PROCEDURE

1. Bring PEG and Diluent reagents to room temperature.

2. Blanks : Dilute 50 ul diluent (B1), standards (SB), and test samples (TB), with 0.95 ml PEG reagent in 2 ml polystyrene tubes, in duplicate.

3. Tests : Dilute 50 ul diluent (B2), standards (S), and test samples (T), with 0.95 ml Antibody reagent in 2 ml polystyrene tubes, in duplicate.

4. Read Blanks and Tests after 15 - 20 minutes at 340nm using the spectrophotometer. Recall programme 4, check volume = 0.8
Zero on PEG reagent. This requires 3 - 4 aspirations before reading stabilises. Read corresponding blanks and then tests for each sample; this will tend to minimise any errors caused by baseline drift.

CALCULATION OF RESULTS

1. Calculate B2 - B1 = OD value for 0 ug/ml albumin
S - SB = OD values for 10 - 200 ug/ml albumin
T - TB = OD values for test samples.

2. Plot a graph of OD 340 values for standards and read test sample results from this; calculate and report mean values.

ACCEPTANCE OF RESULTS

Repeat analysis within batch gives a coefficient of variation of 6%. Patient results should only be reported when QC value is +- 12% of expected value.

REPORTING FORMAT

Results should be reported to the nearest whole number. Reference range based on 62 volunteer subjects and patients with no known organic disease is 0 - 26 ug/ml.

CLINICAL SIGNIFICANCE OF RESULTS

Values greater than 26 ug/ml indicate active inflammatory bowel disease.

INTERFERENCES

The assay is specific for the measurement of albumin in lavage. Albumin is unstable in lavage prior to processing due to presence of proteases in lavage and variable amounts ranging from 10 - 80% of albumin can be lost within 2 hours at 37 C. (based on data from 8 lavages). This limits the sensitivity of the assay.

Gordon Brydon

Clinical Scientist

ALPHA-1-ANTITRYPSIN IN WHOLE GUT LAVAGE FLUID

PRINCIPLE OF METHOD

When human alpha-1-antitrypsin (A1AT) to be assayed reacts with its specific antibody, precipitating immunocomplexes are quickly formed in the presence of polyethylene glycol. If the antibody is present in large excess, these precipitates produce a turbidity which is related to the concentration of albumin in the sample. The turbidity is photometrically measured at the wavelength 340nm. Absorbance readings obtained by assaying calibration standards are used to generate a standard curve, from which the concentration of A1AT in the sample is derived.

REFERENCES

Brydon WG, Choudari CP, and Ferguson A. Relative specificity for active inflammatory bowel disease of plasma derived proteins in gut lavage fluid. Eur J. of Gast and Hepatol 1993, 5:269-273.

HAZARDOUS SUBSTANCES AND NATURE OF HAZARD

CHEMICAL

sodium azide: toxic - see COSSH form no 107
phenyl methyl sulphonyl fluoride: toxic - see COSSH form no 104

BIOLOGICAL

Whole gut lavage fluid (WGLF) is potentially infectious and should be handled appropriately. Procedures for cleaning glassware and disposal of tips which have come in contact with these materials should be strictly adhered to.

PRECAUTIONS

Safety measures as detailed in the Departmental safety handbook must be followed. Appropriate COSHH forms should be read and understood before undertaking this assay.

SPECIMEN REQUIREMENTS

Whole gut lavage fluid: 6 x 1.5 ml samples of whole gut lavage fluid (filtered and processed) should be aliquoted into 2 ml microtubes with screw lids (Sarstedt, UK). Samples should be stored at -70 C. The procedure for filtering and processing lavage fluid is described below.

1. 2 x 25 ml samples of clear lavage fluid are collected from the patient and processed withion 15 minutes where possible. If the collected samples do not appear very clear or do not filter relatively quickly then centrifuge the samples at 3600 rpm for 5 minutes.

2. Filter 10 - 15 ml into a universal container, using Whatman GF/A 12.5 cm filter paper. Transfer 10 ml to another universal container.

3. Add 1 ml SBTI. Mix.
Add 0.56 ml NaEDTA. Mix.
Add 0.24 ml PMSF . Mix.
Add 0.12 ml sodium azide. Mix.

Leave for 2 minutes.

Add 0.6 ml new born calf serum. Mix, and dispense aliquots.

SAMPLE STORAGE AND STABILITY

samples of lavage should be kept stored at -70. These samples are stable for up to 1 year at least.

Turbidity is measured using the PU8640 spectrophotometer (Room 1).

INSTRUMENTATION

Switch on main power. Side of instrument.
Set to ABS mode
Set wavelength to 340 nm
Set delay to 0.01
Set volume to 0.8
Set Temp Off
Store as program 4.

Lavage samples are diluted with PEG reagent using the GILSON DILUGIL V. This is set to sample 0.05 ml and to dispense 0.95 ml.

Assays are carried out using 2 ml polystyrene tubes (LIP Ltd) 64 x 11 mm; code no 30908.

REAGENTS

All reagents are analar grade and obtained from Merck (BDH) unless otherwise stated. These are stored in the fridge in room 1 unless otherwise stated.

a. **FOR PROCESSING WGLF**

1. Phosphate Buffered Saline: (PBS - Sigma) 1 tablet dissolved in 200 ml distilled water to give pH 7.2.

2. Soy Bean Trypsin Inhibitor: (SBTI - Sigma):Dissolve 0.1g per 100 ml PBS. Solid kept at -20 C.

3. Disodium EDTA: Add 11 g to 100 ml distilled water. Dissolve to form a saturated solution. Store at room temperature. Solid stored in room 1 chemical cupboard.

4. Phenylmethylsulphonylfluoride: (PMSF - Sigma) 0.1M in 95% alcohol (IMS). Store solid in room 1 chemical cupboard.

5. Sodium Azide: 2g/100 ml distilled water.

b. **FOR A1AT ASSAY**

1.PEG reagent: 40g polyethylene glycol 6000 (biochemical grade), 6g tris, 2g Tween 20 (Sigma), and 1g sodium azide dissolved in 800 ml distilled water, adjusted to pH 7.0 with dilute hydrochloric acid and made up to 1 litre volume with distilled water.

2. Anti Human A1AT (goat): (Protein Reference Unit - Sheffield). Store frozen in aliquots of 0.7 ml at -20 C.

3. Antibody Reagent: Dilute antisera 1 in 40 with the PEG reagent on the day of assay.

4. Diluent: 9g sodium chloride, 60g PEG 3350 and 1g sodium azide in 1 litre solution distilled water.

CONTROL MATERIAL

STANDARD

1. Standard Serum SPS01: (Protein Reference Unit - Sheffield). Keep at 4 C.

2. A1AT standards: Dilute standard serum with Diluent (Reagent 4) to give the following range of standards : 0,10,20,50,100,200 ug/ml. Prepare fresh on day of assay.

eg for a standard with A1AT 1.52 g/l. Take 0.1 ml standard and dilute it with 0.66 ml of diluent, to give an a1-antitrypsin 200 ug/ml standard. Dilute 0.2, 0.1, 0.05, and 0.05 ml of this with respectively 0.2, 0.3, 0.45 and 0.95 ml diluent to give further standards of 100, 50, 20, and 10 ug/ml.

QUALITY CONTROL

This consists of a WGLF sample from a patient volunteer who has agreed to have his sample tested for HIV and Hep B status.
Store in aliquots of 0.5 ml at -70 C. Stable for up to 1 year at this temperature.

ANALYTICAL PROCEDURE

1. Bring PEG and Diluent reagents to room temperature.

2. Blanks : Dilute 50 ul diluent (B1), standards (SB), and test samples (TB), with 0.95 ml PEG reagent in 2 ml polystyrene tubes, in duplicate.

3. Tests : Dilute 50 ul diluent (B2), standards (S), and test samples (T), with 0.95 ml Antibody reagent in 2 ml polystyrene tubes, in duplicate.

4. Read Blanks and Tests after 15 - 20 minutes at 340nm using the spectrophotometer. Recall programme 4. Zero on PEG reagent. This requires 3 - 4 aspirations before reading stabilises. Read corresponding blanks and then tests for each sample; this will tend to minimise any errors caused by baseline drift.

CALCULATION OF RESULTS

1. Calculate B2 - B1 = OD value for 0 ug/ml A1AT
S - SB = OD values for 10 - 200 ug/ml A1AT
T - TB = OD values for test samples.

2. Plot a graph of OD 340 values for standards and read test sample results from this. Calculate and report mean values.

ACCEPTANCE OF RESULTS

Repeat analysis within batch gives a coefficient of variation of 11 %. Patient results should only be reported when QC value is +- 22 % of expected value.

REPORTING FORMAT

Results should be reported to the nearest whole number. Reference range based on 62 volunteer subjects and patients with no known organic disease is 0 - 19 ug/ml.

CLINICAL SIGNIFICANCE OF RESULTS

Because there is much less A1AT in serum compared with albumin and IgG, it is a less sensitive marker of leakage in inflammatory bowel disease.

INTERFERENCES

The assay is specific for A1AT in lavage. A1AT is stable in lavage for up to 2 hours at 37 C. without processing.

Gordon Brydon

Clinical Scientist

HAEMOGLOBIN IN WHOLE GUT LAVAGE FLUID AND FAECES

PRINCIPLE OF METHOD

Haemoglobin is converted to fluorescing porphyrins by the removal of Fe. Total haemoglobin is determined by reaction with heated oxalic acid:$FeSO_4$ reagent which converts haem to porphyrin without loss of preformed porphyrins. A 3 step purification procedure eliminates other interfering fluorescent materials which may be present.

REFERENCE

Schwartz S., Dahl J., Ellefson M., Ahlquist D.: Clin Chem: 29 (1983) 2061-2067.
Brydon WG and Ferguson A: Lancet: 340 (1992) 1381-1382

HAZARDOUS SUBSTANCES AND NATURE OF HAZARD

CHEMICAL:

ethyl acetate: harmful-see COSSH form no 74
butanol: harmful - see COSHH form 20
oxalic acid: toxic - see COSSH form 187.
acetic acid:corrosive,flammable-see COSSH form no 138.
orthophosphoric acid: corrosive - see COSSH form no 40.
sodium azide: toxic - see COSSH form no 107
potassium ferricyanide : harmful - see COSSH form no 69.
potassium cyanide : toxic - see COSSH form no 68

BIOLOGICAL:

Both whole gut lavage fluid (WGLF) and faeces are potentially infectious and should be handled appropriately. Procedures for cleaning glassware and disposal of tips which have come in contact with these materials should be strictly adhered to.

PRECAUTIONS

Safety measures as detailed in the Departmental Safety Handbook must be followed. Appropriate COSHH forms should be read and understood before undertaking this assay.

SPECIMEN REQUIREMENTS

1. WGLF: 2 x 1.5 ml samples of WGLF (unfiltered) should be aliquoted into 2 ml microtubes with screw lids (Sarstedt, UK). 30 µl sodium azide (2 g/100 ml distilled water) should be added as preservative, and samples stored at -70.

2. Random faecal samples (about 5 g) should be collected into universal containers, and stored at -70.

SAMPLE STORAGE AND STABILITY

Samples of WGLF or faeces should be kept stored at -70, and analysed within 2 months. There is no data on stability of samples beyond this time.

INSTRUMENTATION

Fluorescence is measured using the LS-5B Luminescence Spectrometer (Department of Medicine). Switch on main power (rear of instrument, rhs). Set excitation slit width to 15 nm (lhs), and emission slit width to 5 nm (rhs). Set excitation wavelength to 402 nm and emission wavelength to 600 nm in the following way

1. Press low high reset simultaneously.
2. Press 402 goto EX
3. Press 600 goto EM
   Leave instrument for 5 minutes to stabilise.
4. Place blank sample in cell carrier (nearest the instrument) and press AUTOZERO (light on)
5. Place standard sample in the cell carrier set value eg 680 for 68.0 µg/ml standard and press AUTOCONC (light on).
6. Repeat steps 4 and 5 to finalise calibration.
7. Test samples can now be read.

REAGENTS

All reagents are analar grade and obtained from Merck (BDH) unless otherwise stated. These are stored in room 17.

1. Oxalic Acid Reagent: 4.0 g oxalic acid (analar) is made up to a 10 ml volume with distilled water, and dissolved in a water bath at 100C. 0.31 g FeSO4 is added to the oxalic acid reagent which is returned to 100 C. 0.10 g uric acid and 0.11 g mannitol are then added with mixing, final volume adjusted to 10 ml and the reagent left at 100 C for 15 minutes. The reagent is prepared fresh before use and used as a suspension. Dispose of excess reagent by washing down sink immediately after use.

2. Ethyl acetate/acetic acid 10/1 v/v. Add 50 ml glacial acetic acid to 500 ml ethyl acetate in a fume cupboard. Mix and store in solvent cupboard. Label corrosive/inflammable.

3. 3M Potassium Acetate (294 g/l). Dissolve 29.4 g potassium acetate (BDH GPR) in 100 ml distilled water. Store at room temperature.

4. 3M Potassium Acetate in 1M Potassium Hydroxide (56 g/l). Dissolve 147 g potassium acetate in about 300 ml distilled water, add 28 g potassium hydroxide (fume cupboard), dissolve with stirring and make up to 500 ml with distilled water. Store at room temperature. Label corrosive.

5. N-butanol (Rathburn Chemicals Ltd, Walkerburn). Store in solvent cupboard.

6. 2M H3PO4/Acetic acid 9/1 v/v. Dissolve 68 ml orthophosphoric acid in 300 ml distilled water (fume cupboard). When cool, make up to 500 ml with distilled water and add 58 ml glacial acetic acid. Store in corrosives cupboard. Label corrosive.

7. PEG 4000 reagent: Dissolve 60 g PEG 3350, 9 g NaCl, and 0.2 g sodium azide in 1 litre of distilled water. Use to make Drabkins reagent. Do not keep any excess reagent. Flush down sink with copious water.

8. Drabkins Reagent: (Sigma cat. no. 525-2) 6 vials.

Each vial contains 1 g sodium bicarbonate, 0.2 g potassium ferricyanide, and 0.05 g sodium cyanide.

Take 1 vial and reconstitute to 1 litre with PEG 4000 reagent. Store at room temperature. Label poisonous.

CONTROL MATERIAL

STANDARD

Cyanomethaemoglobin Standard: Dissolve 10 mg haemoglobin (Sigma - H 7379) in 100 ml Drabkins reagent. Leave at room temperature for 15 minutes. To calculate actual haemoglobin concentration read OD at 540 nm against Drabkins reagent (blank).

Molar extinction coefficient (540 nm) = 44.0
Aliquot (0.5 ml) and store at -70. Label poisonous. Stable for up to 6 months.

QUALITY CONTROL MATERIAL

Take 10 mg haemoglobin (Sigma - H7379) and dissolve in 100 ml golytely. Add sodium azide to final concentration 0.02 g/100ml. Aliquot (0.5 ml) into eppendorfs and store at -70° C. Label toxic.

ANALYTICAL PROCEDURE

1. Lavage fluid samples should be frozen at -70°C before assay to allow haemolysis of any intact red cells. After thawing these should be centrifuged at 2000 rpm in the Mistral 3000i (Room 1), and supernatant used for assay.

2. Add 0.4 ml of the oxalic acid reagent to 0.1 ml lavage fluid supernatant, quality control, haemoglobin standard, and blank (golytely) in a 10 ml stoppered quickfit tube. Mix thoroughly and heat at 100°C for 30 minutes in a water bath. Remove stoppers and cool for 2 minutes.

3. Add 1 ml of the 3M potassium acetate reagent, followed by 3 ml ethyl acetate/acetic acid reagent. Vortex mix for 30 seconds. This extracts the porphyrin analytes into the upper organic phase.

4. Transfer 2 ml of the upper organic phase to a 30 ml stoppered quickfit tube and add 0.8 ml butanol and 6.0 ml of 3M potassium acetate in 1M KOH. Mix for 30 seconds. This removes coproporphyrin and other porphrins not derived from haemoglobin haem (contain more than 2 carboxyl groups) - extracted into the lower alkaline aqueous phase.

5. Transfer 1 ml of the upper organic phase to a 10 ml stoppered quickfit centrifuge tube, and add 3 ml phosphoric acid/acetic acid reagent. Mix for 30 seconds. Remove the top layer which contains chlorophyll. Read the fluorescence of the lower acid extract.

6. For instructions in use of the spectrofluorometer see INSTRUMENTATION section.
Tests readings / 10 give results in µg/ml.

7. For faecal haemoglobin, weigh out about 0.1 g faeces, and add whole gut lavage solution to this to make the final faecal concentration 0.1 g faeces per ml lavage fluid. Break up the faeces into a fine suspension using wooden applicators and rotaxixer. Use 0.1 ml faecal suspension (= 10 mg faeces) as sample and then analyse as for lavage.

spectrofluorometer readings / 1000 give results in mg haemoglobin per g faeces.

NOTE Please retain all waste from extractions into a 500 ml reagent bottle and periodically remove ethyl acetate/butanol upper phase to solvent waste bottle. Discard lower phase down sink.

CALCULATION OF RESULTS

For WGLF test readings /10 give results in ug haemoglobin /ml WGLF.
For faeces test readings /1000 give results in mg haemoglobin /g faeces.

ACCEPTANCE OF RESULTS

The coefficient of variation (between batch - reproduceability) of quality control material currently gives a value of 8%.
With a between batch variation of 8%, 2 SDs = 16% of mean value.
ie if mean value is 135 µg/ml, acceptable values will lie between 113 and 157 µg/ml. If the value lies outwith this value the analytical run should be repeated.

REPORT FORMAT

The method for lavage haemoglobin is sensitive to 0.5 µg/ml, and results should be reported to 1 µg/ml.
For faecal haemoglobin results should be reported to 0.1 mg/g
Reference values for lavage haemoglobin range from 0 - 5 µg/ml.
(Brydon and Ferguson 1992).
Reference values for faecal haemoglobin range from 0.1 - 2.0 mg/g

CLINICAL SIGNIFICANCE OF RESULTS

The reference range for WGLF haemoglobin is 0-5 ug/ml which is equivalent to daily blood loss of 1ml. Values in excess of this indicate pathological GI blood loss.
The reference range for faecal haemoglobin is 0.1 - 2.0 mg/g faeces. Values in excess of this indicate pathological GI blood loss.

INTERFERENCES AND LIMITATIONS

Other haem containing proteins have the potential to interfere in this assay; eg myoglobin in meat, peroxidases in vegetables, although neither of these should cause significant interference in lavage samples; lavage still contaminated with faecal material, where any blood cells may be concentrated should be avoided. Faecal haemoglobin values can be raised by up to 1 mg/g faeces for a daily red meat intake of 100 g, given an average stool output of 150 g. Vegetable peroxidases will cause no significant interference taken even in large amounts.

Gordon Brydon

Clinical Scientist
1st June 1995

INSTRUCTIONS FOR USE OF LS-3B LUMINESCENCE SPECTROMETER

1. Switch on main power (rear of instrument, rhs).

2. Set excitation slit width to 15 nm (lhs), and emission slit width to 5 nm (rhs).

3. Set excitation wavelength to 402 nm and emission wavelength to 600 nm in the following way

    1. Press low high reset simultaneously.

    2. Press 402 goto EX

    3. Press 600 goto EM
    Leave instrument for 5 minutes to stabilise.

    4. Place blank sample in cell carrier (nearest the instrument) and press AUTOZERO (light on)

    5. Place standard sample in the cell carrier set value eg 680 for 68.0 µg/ml standard and press AUTOCONC (light on).

    6. Repeat steps 4 and 5 to finalise calibration.

    7. Test samples can now be read.

GAMMA/DELTA INTRA EPITHELIAL T CELLS IN SMALL INTESTINAL BIOPSIES: QUANTIFICATION BY IMAGE ANALYSIS

TEST PRINCIPLE

Image analysis can be used to identify parameters such as size,number,shape,position and optical density of identifiable parts of an image.
The Tas + image analyser utilises a microscope,video camera,visual display monitor,dec computer with double disc drive and a printer.The computer software operates on the Tasic programming language.
An image from the microscope is transferred by the video camera to the monitor.The image on the screen is then altered to give an image suitable for measuring (image preprocessing),here the image is converted into a binary image where different grey intensities can be detected.
The image analyser can now measure the areas that are detectable and record the value on the printer.

REFERENCES

Leitz tasic + reference manual
Joyce-Loebl: Image analysis principles & practice
E.ARRANZ et al.Candidate markers of potential coeliac disease.unpublished data.

CHEMICALS AND EQUIPMENT REQUIRED

TAS + IMAGE ANALYSIS SYSTEM
fitted with good quality microscope *10 and *40 objectives.

Stage micrometer

SAMPLES

Cryostat sections that have been fixed in acetone and stained immunohistochemicaly using monoclonal antibodies that detect CD3 or gamma delta expression.

INSTRUMENTATION

IMAGE ANALYSER

1.Remove dust covers

2.Switch on at wall

3.Insert Tasic 8" floppy disk in drive 0 (left hand drive)

4.Insert G-I lab disc in drive 1

5.Switch on with the key

6.Enter date on prompt,input the date followed by a dash,then the first 3 letters of the month followed by a dash and then the last two digits of the year,eg 31-JUL-93.Press return.

7.Set up microscope

MICROSCOPY

For accurate quantification it is essential that the microscope is set to give optimum resolution and even illumination.The best way to achieve this is by setting the microscope to give Kohler Illumination.

A.Set lamp brightness on the control box to a comfortable intensity by pressing light switch followed by the * button.

B.Divert the light to the microscope eyepieces,use the pull push rod at the top right hand side of the microscope.

C.For *10 objectives and below remove flip top condenser.

D.Fully open the field diaphram (bottom of microscope).

E.Fully open the condenser diaphram (beside the flip top condenser)

F.Place a well stained section on microscope stage.

G.Focus on the section using the coarse focus knob,adjusting the focus in the eyepieces so that both eyepieces are in equal focus.

H.Fully close the field diaphram.

I.Rack the condenser up and down until a sharp image of the field diaphram is obtained.

J.Centre the image with the centring screws on the condenser.

K.Open the field diaphram until it is just outside the field of view.

L.Remove the left eyepiece.

M.Close the condenser diaphram until it occupies one third of the light coming through the eyepiece tube.

N.Repeat the set up procedure when changing to a new objective.

CALIBRATION OF IMAGE ANALYSER

Place stage micrometer on the microscope stage and focus. Switch light to video camera and refocus using control box. Set focus on and use the arrow keys to focus up and down.
Line up the edge line of the box on the screen with the zero line of the stage micrometer,use control box press stage button followed by arrow keys.The distance from one edge of the box to the other can now be measured and the distance input into the image analyser for calibration.

The following calibration figures can be used for the present set up.

Objective	Calibration(microns)
*4	1500
*10	600
*25	233
*40	148
*100	60

7.Set calibration by typing TSC148 if using *40 objective. For other objectives type in TSC followed by the calibration figure.
Press return.

8.Type /U,DY1:CD3TCR.OCT
Press return.

9.Type /E press return.

10.Type in the sample number on the prompt,press return.

11.Select the field to be measured and then press return.

12.Turn up screen brightness and draw a line along the length of epithelium you wish to measure.

13.Touch sensefield 20 with the light pen.

14.You should now touch your drawn line with the light pen.

15.The length of epithelium will now be printed on the screen.

16.On the screen the prompt will ask you to input the total number of +ve cells.Type in the number of +ve cells in the epithelium of the area measured and press return.

17.The number of +ve cells will now be printed on the screen.

18.On the screen the prompt will ask you "do you wish another field yes or no?"

If you type y the new field number will be entered on the screen and you will be ask to select new field (instruction 10).
If you type n the printer will print out the final measurements including the total number of cells,the total area of lamina propria and the mean number of cells/sq.mm.

COUNTING PROCEDURE

The section is initially examined under a low power in order to assess its suitability for counting.After initial examination the *40 objective is selected. Starting at one end of the specimen the epithelium is followed along its length. Lengths of epithelium that are traumatised are not measured or counted. A minimum of twenty fields should be counted.

REPORT FORMAT

The results are reported in whole numbers as the number of positive cells per mm.Only specimens where the length of epithelium exceeds 3mm are reported except where discussion with senior scientists and medical staff deem a lesser specimen reportable.
A senior member of the medical staff should place there comments on reports and an indication of whether the result falls inside or outside the reference range noted.
The results of all counts are stored in printouts and recorded on the cryo floppy disk located in room 20.Results include length of epithelium,cell numbers counted,% of gamma delta cells to cd3 cells,pathology reports and biopsy numbers and diagnosis when available.

REFERENCE RANGE

The reference range for CD3 is < 68 per mm

The reference range for gamma delta cells is < 5.5

John Bode

Chief MLSO
17th August 1995

CD3 LYMPHOCYTES IN SMALL INTESTINAL EPITHELIUM

TEST PRINCIPLE

CD3 + intra epithelial T lymphocytes are generally increased in patients with coeliac disease. These cells can be detected by immunocytochemistry using the antibody monoclonal anti-CD3 on cryostat sections and quantified using image analysis.

Non specific antibody interaction is blocked by treating the section with diluted normal serum of the host producing the secondary antibody. Monoclonal antibody directed against CD3 is placed in contact with the tissue section and allowed to react.

The secondary biotinylated antibody which is directed against the bound monoclonal antibody is then placed on the tissue section and allowed to react.

Finally the streptavidin biotin peroxidase is added and this binds to the biotinylated secondary antibody.

The procedure is then visualised histochemicaly by reacting the peroxidase with the peroxidase substrate diaminobenzidine.

The presence of endogenous peroxidase makes this method unsuitable for Lamina Propria evaluation of +ve cells.The removal of peroxidase in the tissue section may be detrimental to the demonstration of this particular antigen.

REFERENCES

J.SPENCER et al .Gamma/delta T cells and the diagnosis of coeliac disease. Clin Exp.Immunology (1991) 85,109-113
E.ARRANZ et al. Candidate markers of potential coeliac disease.
unpublished data.

HAZARDOUS SUBSTANCES

BIOLOGICAL

Unfixed tissue is potentially infectious and should be handled appropriately.

CHEMICAL

Chemicals and serum should be handled in accordance with the appropriate COSHH forms.The laboratory safety handbook procedures should be adhered to.

Cryospray Brights order code B1029; COSHH form no 155

Industrial Methylated Spirit (IMS99); COSHH form no 173

Acetone BDH analar grade product ; COSHH form no 139

Hydrogen Peroxide BP 6% (20 volume) Hilcross Pharmaceuticals ; COSHH form no 171.

3,3'-Diaminobenzidine Sigma COSHH form no 158.

Imidazole Sigma; COSHH form no 172

DePeX COSHH form no 157

Hematoxylin Gills NO1 Sigma; code GHS-1-80; COSHH form no 169 :room 16

PATIENT PROCEDURE

see procedure for collection of jejunal biopsy.

SAMPLE COLLECTION , STORAGE, AND STABILITY

The monoclonal antibody is not suitable for specimens which have been processed to paraffin wax.
Cryostat sections can be used following brief fixation in acetone.
Crosby capsule or endoscopic biopsies are collected unfixed as soon after biopsy as possible.
Tissue is placed in a drop of OCT on a glass slide and, under the dissecting microscope, is orientated.
The villous surface should be uppermost and the tissue flat.
The tissue is then rapidly frozen,wrapped in tin foil and stored in the -70 freezer.

REAGENTS

SERUM AND ANTIBODIES

Rabbit serum SAPU;code S030-220 batch NO 6208T
room 16 fridge

monoclonal anti CD3 ;SAPU; cat NO TA 1061
room 16 fridge

Biotinylated Rabbit anti Mouse immunoglobulins Dako; room 16 fridge

StreptABComplex/peroxidase kit Dako code lot
room 16 fridge

OTHER REAGENTS

Poly-l-lysine 0.1%w/v Sigma code P 8920

Tris BDH analar code 10315
room 16

0.9% Sodium chloride Baxter health care (Pharmacy WGH)code F7124
downstairs coldroom

Histoclear
room 16

Pap pen Dako code 052002
room 16 Finpippete drawer

STOCK REAGENTS

0.01% Poly-l-lysine

Dilute Poly-l-lysine solution 1/10 with distilled water
store in fridge for up to 3 months.

0.5M Tris buffer pH 7.6

Dissolve 60.72gms of Tris in 500mls of Distilled Water adjust pH to 7.6 with 1N Hydrochloric Acid and make up volume to 1ltr.
Store room 18 fridge

WORKING REAGENTS

1. 70% Industrial methylated spirit

IMS 70mls
Distilled water 30mls

2. 0.05M Tris buffered saline pH 7.6 (TBS)

dilute 0.5M tris buffer 1/10 with 0.9% sodium chloride

3. Normal Rabbit serum/TBS (NRS/TBS)(blocking serum)

Rabbit Serum : 1ml
TBS 0.05M pH 7.6 : 4mls

4. Antibody dilutions

a. anti CD3 1/20 in NRS/TBS(primary antibody)

b. Biotinylated Rabbit anti Mouse 1/300 in NRS/TBS(secondary antibody)

c. Dako ABC peroxidase complex(label)

9ul A
9ul B
1ml TBS
prepare at least 1/2 hr before use.

SUBSTRATE

1 0.5M tris pH 7.6	10mls
2 Diaminobenzidine	10mg
3 Imidazole	1 flake
4 6% Hydrogen peroxide	60ul

Prepare fresh

CONTROL SECTIONS

The lymphocytes in the lamina propria act as a +ve control.

ANALYSIS PROCEDURE

SECTIONING OF FROZEN TISSUE

The tissue is transferred to the cryostat - **DO NOT ALLOW TO THAW** - and bissected. Half of the specimen is embedded at right angles in OCT and then frozen onto a cryostat specimen holder.
6micron Sections are cut at a temperature of -15c TO -20c.The sections are placed on 0.01% Poly-L-Lysine coated slides and allowed to dry at room temperature for 30 minutes to 4 hours.The slides are then wrapped in tin foil and stored in the -20C freezer.

FIXATION OF CRYOSTAT SECTIONS

Sections are removed from the freezer and allowed to rise to room temperature before removal of tin foil.The sections should be thoroughly dry with no traces of moisture on the slides.Sections can now be fixed in fresh analar Acetone for 30 minutes at room temperature.After fixation sections are allowed to dry at room temperature for 30 minutes.A wax pencil or a PAP pen can now be used to draw round the sections to form a reservoir for the antibody solutions.

STAINING METHOD - CD3

1 Place sections in TBS pH 7.6	5 minutes
2 normal rabbit serum/TBS 1:4	10 minutes
3 drain but do not let section dry.	
4 mouse monoclonal anti CD3 dilution 1/20 in TBS/NRS	60 minutes
5 rinse sections in a stream of TBS	
6 wash in TBS	2/5 minutes
7 remove excess TBS but do not let sections dry	
8 biotinylated rabbit anti mouse IgG dilution 1/400 in TBS/NRS	60 minutes
9 rinse sections in a stream of TBS	
10 wash in TBS	2/5 minutes
11 remove excess TBS but do not let sections dry	
12 streptavidin/biotin peroxidase complex in TBS	60 minutes
13 rinse sections in a stream of TBS	
14 wash in TBS	2/5 minutes
15 treat sections with DAB	10 minutes

16 wash in running tap water		10 minutes
17 stain in haematoxylin		1 minute
18 wash in running tap water		15 seconds
19 saturated aqueous lithium carbonate	till "blue"	
20 wash in running tap water		1 minute
31 70% IMS		10 seconds
32 IMS		15 seconds
33 isopropanol		20 seconds
34 histoclear		1 minute
35 histoclear		5 minutes
36 mount sections in DPX		

QUANTIFICATION

+ve cells,endogenous peroxidase	brown
nuclei	blue

the addition of 5% human serum to the secondary antibody can help be helpful in reducing non specific background staining.

Cell counts and mucosal length measurment are carried out using the TAS+ Image Analyser.

Only +ve staining cells in the epithelial cells of the villi are counted and the length of mucosa from the counted area measured.The measurements are carried out at *40 magnification using the program CD3TCR.OCT.

REPORT FORMAT

The results are expressed as the number of +ve cells/mm of tissue.
The normal range is jejunum <68/mm

John Bode

Chief MLSO
17th August 1995

ELISA ASSAYS

ELISA = Enzyme linked immunosorbent assay

TECHNIQUE PRINCIPLE AND METHOD OUTLINE

Non competitive ELISA assays are used to measure total or antigen specific Immunoglobulins G, A or M in serum, lavage or aspirate materials. Antibodies commonly measured those to gliadin (wheat protein), ovalbumin (egg protein) and lactoglobulin (milk protein).

A double antibody sandwich technique is used for total immunoglobulin quantitation against a purified secondary standard material. An indirect technique is used for estimation of antigen specific immunoglobulins measured against a known high human sample.

Class specific antihuman immunoglobulin or pure food antigen is bound in excess to a solid phase and then washed. In these methods, a 96 well ELISA plate is the solid phase - different plate types have different binding characteristics.
The plates are incubated with a protein containing solution to "block" any non specific binding sites.
Samples for total immunoglobulin quantitation are added to the plates in doubling dilutions - in order to cover the wide range of concentrations found while samples for antigen specific analysis are added at a single predetermined dilution. Samples are incubated and then washed. Binding takes place between the antihuman globulin and the immunoglobulin being quantified or between the specific antigen and corresponding antiantigen immunoglobulin.
A class specific antihuman antibody conjugated with alkaline phosphatase is added to the plate, incubated and then washed. This conjugated antibody binds to the complex bound to the solid phase.

The substrate for alkaline phosphatase is p-nitrophenyl phosphate in a diethanolamine uffer at pH 9.8. The substrate is added to the plate and the reaction

p-nitrophenyl phosphate ---------- p-nitrophenol
colourless yellow

takes place. The colour development takes place within an hour and the optical density at 405 nm is read on a dedicated spectrophotometer/ELISA reader, a Dynatech MR5000. The colour development can be monitored and the absorbances measured when the top standard or reference standard has reached a previously determined value.

Results can be calculated either manually, using a programme on the ELISA reader or a Minitab programme..

ELISA REAGENTS (1)

Updated .....

SPS-O1 - standard material for quantifying IgG and IgM. Purchased in 5 x 1 ml vials from Dept. of Immunology, P O Box 894, Sheffield S5 7YT.
Store at 4 °C in fridge A. Date bottle on opening and discard after 1 month.

Current batch .....

Total IgG conc ..... **g/l**

Required top std for IgG assay in lavage is 250 ng/ml

Dilute 10µl SPS-O1 in ..... µl diluent to give conc 100 µg/ml
Dilute 10µl diluted SPS-O1 (100µg/ml) to 4 ml to give conc 250 ng/ml

Total IgM conc ..... **g/l**

Required top std for IgM assay in aspirate is 1000ng/ml

Dilute 10 µl SPS-O1 in ..... ml to give conc 1000 ng/ml

Human IgA - purified immunoglobulin from colostrum. Standard material for quantifying IgA in secretions. Purchased in 1 ml vial from Sigma Chemical Co., Cat No I-2636

Current batch .....

Total IgA conc ..... **g/l**

1 ml conc (neat) soln diluted to 10 ml to give conc ..... µg/ml

110 µl aliquots at ..... µg/ml stored at -20°C in freezer B

Required top std for IgA assay in aspirate = 1000ng/ml

Dilute 100 µl fresh aliquot in ..... ml to give conc 1000 ng/ml

ELISA REAGENTS (1 cont)

Coating antibodies

Goat antihuman IgG, Fc specific, Affinity purified, purchased from Sigma Chemical Co. (Cat No I2136)

Current batch ..... Use at 1 : ..... dilution

Goat antihuman IgM, μ chain specific, purchased from Sigma Chemical Co. (Cat No I2386)

Current batch ..... Use at 1 : ..... dilution

Goat antihuman IgA, α chain specific, purchased from Sigma Chemical Co (Cat No I2261)

Current batch ..... Use at 1 : ..... dilution

Conjugated antibodies

Goat antihuman IgG, alk phos conjugated, purchased from Sigma Chemical Co (Cat No A9544)

Current batch .....

For Antigliadin ELISA Use at 1: .....dilution
For Total IgG ELISA Use at 1: .....dilution

Goat antihuman IgM, alk phos conjugated, purchased from Sigma Chemical Co (Cat No A3437)

Current batch .....

For Total IgM ELISA Use at 1: .....dilution

Goat antihuman IgA, alk phos conjugated, purchased from Sigma Chemical Co (Cat No A3036)

Current batch .....

For Antigliadin ELISA Use at 1: .....dilution
For Total IgA ELISA Use at 1: .....dilution

ELISA REAGENTS (2)

All solutions must be dated and signed.

Sterile Water

1 litre sterile water containers (Baxter Healthcare Code F7114) are stored at 4°C in the cold room.

Coating buffer

Carbonate-bicarbonate coating buffer, 0.05M, pH 9.6 at 25°C. Dissolve the contents of 10 capsules (Sigma Chemical Co. Cat No C-3041) in 1 litre sterile water. Mix to dissolve and store at 4°C in fridge A.

Sodium chloride

0.9% sodium chloride in 1 litre sterile containers (Baxter Healthcare Code F7124) is stored at 4°C in the cold room.

Wash Solution

0.9% saline + 0.05% Tween 20 is the wash solution used for both manual washing of ELISA plates and for the Dynatech MRW ELISA automatic washer.
Add 500 µl Tween 20 (Polyoxyethylene-Sorbitan Monolaurate, Sigma Chem Co. Cat No P-1379) to 1 litre of 0.9% saline. Mix and store at 4°C in fridge A.

Adult Bovine Serum

Adult bovine serum is purchased from SAPU, Law Hospital, Carluke, Lanarkshire, ML8 5ES (Product No S026-220) in 20 x 20 ml aliquots and kept frozen at - 20°C in Freezer B. Allow an aliquot to reach room temperature and then filter through a 22u filter before using to constitute the **Diluent**. The second 10 ml of the aliquot can be stored at 4°C in Fridge A for 1 week before use.

Diluent

0.9% saline + 0.05% Tween 20 + 1% adult bovine serum.
Add 500 µl Tween 20 and 10 ml filtered adult bovine serum to 1 litre of saline. Mix and store at 4°C in fridge A.

Diethanolamine (DEA) substrate

5 litres of DEA substrate is constituted in a semi quantitative flask.

Add:
500 ml diethanolamine - concentrated liquid (purchase 4 x 500 ml from BDH Laboratory Supplies, Poole, BH15 1TD. Analar Reagent Product No 10393 4J) stored in the flammable cupboard at room temperature in the chemical store.
0.51 g magnesium chloride ($Mg\ Cl_2 6H_2O$)
1.0 g sodium azide (NaN_3)
4 l sterile water

Adjust the pH to 9.8 with 6N hydrochloric acid (HCl) - kept in Room 1 Chemical cupboard and make up to 5 l with sterile water.

Store in 1litre bottles (ex sterile water) at 4°C, 1 currently in use in Fridge A and the remainder in the Cold Room.

P-nitrophenylphosphate

5 mg phosphatase substrate tablets - disodium p-nitrophenyl phosphate hexahydrate. Purchase 8 x 200 tablet bottles from Sigma Chemical Co Product no 104-105. Tablets are stored at -20°C in freezer B.

Alkaline phosphatase substrate

Fresh substrate is constituted aprox 30 mins before required. Dissolve 1 tablet phosphatase substrate per 5 ml DEA buffer, mix thoroughly.

INSTRUMENTATION (Room 13)

Dynatech Multi-Reagent Washer

The ELISA plate washer is programmed for several (usually 3) wash cycles followed by an optional fill with blocking solution.
The instructions for the use of this instrument are on the adjacent wall and in the Room 13 Equipment Folder. Instructions must be followed carefully with particular attention paid to decontamination procedures.

Denley Wellmixx1

The Wellmix can shake 1 or 2 ELISA plates automatically.
It is switched on at the wall socket and by rocker switch at the front of the shaker.

Dynatech MR5000 Microplate Reader

The microplate reader is used in the MANUAL mode in order to find the current absorbance of samples and in this mode is used to determine the end point of the alkaline phosphatase development reaction.
The microplate reader is also used in a programme mode - set at the appropriate programme for the analysis being carried out - to measure the final absorbance and if required to use the absorbances to calculate the results of the assay.
The instrument is programmed via the touch sensitive front panel.

1. Switch on the microplate reader (power switch at back of instrument) and attached printer. The instrument runs a self check (about 10 s) then shows **READY**.
2. Place plate on the plate carrier.
3. Press **MANUAL**
4. Wavelength mode ? Press **DUAL**
5. Filter - set to 405nm, **ENTER**
6. Ref. Filter - set to 630 nm, **ENTER**
7. The instrument will now read the absorbances of the plate and hold the results so that the absorbance at each plate position can be viewed by using the directional arrows.

8. When the (top) standard has reached the required value read the plate on the corresponding test programme.

9. Press **START**
10. Test number = Enter programme no. **ENTER** to confirm correct assay programme.
11. Plate = using direction arrows and/or number keyboard enter any combinations of letters or numbers to identify the plate.
12. The absorbances will now be read and printed out in template pattern.
13. If only absorbances are required **ESC.**
    If calculations are required refer to the method of the specific assay.

HAZARDOUS SUBSTANCES

Precautions

The Departmental Safety Handbook and the relevant COSSH forms must be read before undertaking any assay.

Chemical hazards:

1. Diethanolamine (DEA): toxic, irritant - see COSSH form # 70
2. P-nitrophenylphosphate tablets: harmful - see COSSH form # 76

Biological hazards

All patient material, and standard or control material of animal origin, must be regarded as potentially infectious and handled accordingly. Manually washing of plates containing biological material must be by vacuum into a sealed waste trap. Biological waste from manual washing or using the Multi-Reagent Washer must be decontaminated using Presept tablets before discarding down the sink.The disposal of tips and tubes must be as recommended in the laboratory safety handbook.

ESTIMATION OF ANTIGLIADIN IgA AND IgG IN SERUM

TEST INDICATIONS

Serum IgA and IgG antigliadin antibodies (AGA) are measured in patients to support a diagnosis of coeliac disease and in patients in whom this diagnosis has been confirmed to follow the compliance of a gluten free diet.

TEST CONTRAINDICATIONS

Serum IgA and IgG AGAs are not helpful in diagnosis if the patient is already on a gluten free diet.
Serum IgA AGA is not indicated in patients with IgA deficiency and in cases with inappropriately low levels of serum IgA AGA a general IgA deficiency should be considered.

TEST PRINCIPLE

Serum IgA and IgG AGA concentrations are routinely measured against a precalibrated human serum in a dilution series using an ELISA technique.

REFERENCES

Unsworth D.J., Leonard J.N., McMinn R.M.H., et al. Br. J. Dermatol. 1981 **105** 653-8
Anti-gliadin antibodies and small intestinalmucosal damage in dermatitis herpetiformis.

O'Mahoney S., Arranz E., Barton J.R., Ferguson,A. Gut 1991 **32** 29-35
Dissociation between systemic and mucosal immune resposes in coeliac disease.

PATIENT PROCEDURE

There are no specific conditions pertaining to the collection of blood samples for this assay. Details of the patient's diet should be obtained.

SAMPLE STORAGE AND STABILITY

10 mls blood are drawn from a patient using a monovette (z/10) and needle and allowed to clot at room temperature for 1 hour.
The blood sample is centrifuged at 2500 rpm for 10 minutes then the serum separated into storage tube(s) following sample processing procedures. *(Refer to Protocols File)*
Samples are stored at -20°C in the freezer in Room 1.
Serum or plasma can be used in this assay. *(Refer to Method Development file for validation of the use of plasma)*

Samples are stable for up to 1 year if stored as above.

STANDARD AND QUALITY CONTROL MATERIAL

A patient who is expected to have raised serum AGA levels is asked to give consent to have a large blood sample taken and tested for Hepatitis and HIV infectivity. A 50 ml blood

sample is taken, separated, an aliquot is sent for testing and the remaining serum stored at -20°C. If this sample is negative for infectivity and the AGA value is appropriate the sample is calibrated against the current standard or control material and then divided into 100µl aliquots in small Eppendorf tubes and stored at -70°C in Freezer 2.

Two QC material are used, 1 within the reference range (Lo QC) and 1 above the reference range (Hi QC). A Lo QC can be produced from a sample with a high level by dilution with a tested sample of very low level. New QC material must be run in parallel with current to establish confidence limits.

(Refer to QC documentation in the Protocol file for details of the current materials used)

ANALYSIS PROCEDURE

1. Coat plate with appropriate GLIADIN solution. Use IMMULON 2, flat bottomed, gamma irradiated plates for these assays. Prepare a template to mark the sample positions. GLIADIN - gliadin extract (Sigma G-3375) is stored at room temperature in Room 13. Weigh out 5mg antigen and dissolve in 10 ml Coating Buffer using a magnetic stirrer to give 0.5 mg/ml solution. Further dilute 1 ml of this stock in 20 ml of coating buffer to give a coating solution of 25 µg/ml.
2. Using an 8 channel multichannel pipette, dispense 100 µl coating solution to each well of the ELISA plate.Cover plate with a plastic lid, place in a moist box and incubate at 22°C for 5 hours.

3. Block plate
   Either manually or using the Dynatech M-RW wash the plate x 3 with wash solution, and then add 250 µl Diluent. Replace plate in moist box at 22°C and leave for a minimum of 2 hours.
4. At this stage the plate may be vacuum sealed into a plastic bag and stored at -20°C until required. Several plates can therefore be prepared at the same time, stored, taken out individually as required. Plates stored in this manner are stable for at least 3 months. Allow to reach room temperature before proceeding to the next stage.

5. Prepare standards
   IgA - dilute standard material in Diluent - currently = Main @ 1/100 (add 10µl standard to 990µl Diluent), next = sample 95/1548 @ 1/467 (add 3µl standard to 1398µl Diluent).
   IgG - dilute standard material in Diluent - currently = Main @ 1/250 (add 4µl standard to 996µl Diluent), next = sample 95/1548 @ 1/1115 (add 3µl standard to 3342µl Diluent).
6. Prepare samples
   IgA - dilute QC samples and test samples 1/1000 in Diluent (add 3µl sample to 2997µl Diluent).
   IgG - dilute QC samples and test samples 1/2000 in Diluent (add 2.5µl sample to 4997.5µl Diluent) or alternatively further dilute the sample dilution for IgA 1/2 in the plate (add 50µl 1/1000 sample to 50µl Diluent in each well).

7. Load standards, QC material and sera on to the plate
   Dispel the blocking solution from the plate and blot dry on absorbant paper.
   Using the 8 channel multipipette, dispense 100µl Diluent into wells A1, A2, and C1&2 to H1&2.
   To B1, B2, C1 and C2 add 100µl of the appropriate standard. Mix the standard and diluent in C1, C2 by drawing up and down the pipette tip 3 times. Transfer 100µl to D1, D2, mix, and so on down the plate to prepare a doubling dilution series.
   Load 100µl of QC samples and serum samples into duplicate wells as planned on the template.

8. Cover plate with a plastic lid and incubate the plate at 4°C overnight in a covered moist box.

9. Conjugate
   Prepare alk.phos conjugated antisera dilutions in Diluent following dilutions on page 4.
10. Wash plate as above, then using an 8 channel multipipette dispense 100 µl of the appropriate conjugate to each well of the ELISA plate.
11. Cover plate with a plastic lid, place in a moist box and leave at 22°C for 5 hours.

12. Colour Development
    Prepare alkaline phosphatase substrate (15 mls per plate) no more than 15 mins before it is required.
13. Wash plate as above, then using an 8 channel multipipette dispense 100 µl substrate to each well of the ELISA plate. Leave on the bench as the colour starts to develop and only shake on Wellmix if colour development is very slow.
14. Monitor the colour development manually on the Microplate Reader and as the appropriate standard reaches its endpoint **START** the programme.

    Absorbance endpoint for IgA = when top standard reads about 1.00
    Absorbance endpoint for IgG = when 2nd standard reads about 1.00

15. Programme for antigliadin assay = 43 "GLIADIN". The printout automatically subtracts the blank value (A1 and A2).

CALCULATION OF RESULTS

Plot the duplicate standard absorbance values as y co-ordinates on Inplot or Prism graph programmes, using the following values as x co-ordinates:
IgA top standard = 1.0 Handy Units, 2nd standard = 0.5 etc
IgG top standard = 0.8 Handy Units, 2nd standard = 0.4 etc
Plot the best curve fit through these points using non-linear regression.
Mean the duplicate absorbance values for each QC and test.
Using the appropriate command, calculate the corresponding results in Handy units from the standard curve for all the QC and test absorbances, remembering to multiply the result read off the curve by the appropriate dilution factor (e.g. 1000 for IgA).

ACCEPTANCE OF RESULTS

The test results are technically acceptable if the QC absorbance values lie within 95% of the current QC absorbance values and if the duplicate values differ by no more than 10%.

REPORT FORMAT

Results are entered into the REPORTS database reported to the nearest whole number, and printed via the Mailmerge programme.

Serum antigliadin IgA = ... units
(Reference range 0 - 30 units)

Serum antigliadin IgG = ... units
(Reference range 0 - 45 units)

CLINICAL SIGNIFICANCE OF RESULTS

Results are assessed in conjunction with IgA antiendomysium antibody results.
Serum IgG AGA levels increase with age and a single raised IgG AGA result is not usually clinically significant.
Since a definitive diagnosis is still dependent upon a histological assessment, if IgA and IgG AGA and IgA AEM antibodies are all raised then a jejunal biopsy is recommended.

METHOD VALIDATION

Performance characteristics

Antigliadin IgA
Within batch CV = 9.4% at 20 units
= 7.5% at 90 units
Between batch CV = % at units
= % at units

Antigliadin IgG
Within batch CV = 9.9% at 26 units
= 5.9% at 117 units
Between batch CV = % at units
= % at units

STANDARDISATION

When a new standard serum is required the next sample from a likely patient (see Standard and Quality Control Material section) should be run on the assay several times to ascertain the sample's unitage. The potential standard should then be run in a doubling dilution series, starting with dilutions which should give a result of 1.0 units for IgA or 0.8 units for IgG, alongside the current standard curve. 3 such curves should be run using starting dilutions around the calculated value, and the curves plotted on top of the current standard curve. The set of dilutions which best fits the current standard curve should be chosen. If none is a good fit then the excercise should be repeated with different starting dilutions, according to which way out the curves were.

For example:
IgA - Main - top standard of curve (1/100 dilution) = 1.0 units.
95/1548 was run several times giving an average result of 471 Handy units in the serum. Thus a dilution of 1/471 of the serum should produce a solution containing 1.0 units to be used as the top standard. In practise 1/467 was actually used.
So 95/1548 was run in dilution series starting with 1/400, 1/467 and 1/500 as the first dilutions. These were run alongside Main standard curve. The 1/467 curve fitted exactly over the Main curve, and furthermore samples which were read off from both curves gave almost identical results at high and low levels.
IgG - top standard = 0.8 units, therefore a sample with 892 units in would need a 1/1115 dilution to produce a solution containing 0.8 units. So this plus two other dilutions (e.g. 1/1050 and 1/1200) should be compared with the original curve.

ESTIMATION OF TOTAL IgG IN LAVAGE

TEST INDICATIONS

Patients may undergo bowel cleansing prior to endoscopic procedures or surgery or at the start of an elemental diet. The whole gut perfusate (WGL) obtained after the removal of all faecal material contains any biochemical compounds which have been secreted or leaked into the gut. The concentration of total IgG (and also albumin and α1-antitrypsin) in WGL fluid are raised in patients with active gut inflammation.

Total IgG is therefore measured in WGL to assist in the diagnosis of inflammatory bowel disease and in patients with confirmed disease as an alternative/additional measure of the degree of inflammation.

TEST CONTRAINDICATIONS

Bowel preparation using WGL fluid may not be appropriate in all patients.

The measurement of proteins in WGL fluid is only useful if the WGL has been strictly collected and treated according to the protocols.

TEST PRINCIPLES

Total IgG in lavage is quantified against the protein reference standard (SPS-01).in doubling dilutions using an Elisa technique.

REFERENCES

Gaspari M.M., Brennan P.T., Soloman S.M. Elson,C.O. J. Immunol. Methods 1988 **110** 85-91 A method of obtaining, processing and analysing human intestinal secretions for antibody content.

O'Mahoney S., Choudari C.P., Barton J.R., Walker S., Ferguson A. Scand.J. Gastroenterol. 1991 **26** 940-944 Gut lavage fluid proteins as markers of activity in inflammatory bowel disease.

PATIENT PROCEDURE

Hospital inpatients or outpatients must present for bowel preparation fasting.

Patients are required to drink aproximately 4 litres of *Klean-Prep* -a polyethylene glycol electrolyte solution- at a rate of 1 glass (250 mls) every 10 minutes. Alternatively the liquid may be given via a naso-gastric tube.

After aproximately 2 hours the patient passes loose watery stools which, on continued drinking, becomes a clear yellow liquid. When the rate of influx is standardised , this becomes essentially a gut perfusate. 60 mls of this clear fluid is collected for processing.

SAMPLE STORAGE AND STABILITY

IgG in untreated lavage material is stable for 2 hours at 37°C However since the material is usually required for the estimation of other more labile parameters it must be processed and stored immediately after collection.

Samples are processed as directed in the lavage processing protocol and stored at -70°C. The processing required and the volumes stored may vary depending upon the current requirements. Aliquots of the same sample are stored in both -70°C freezers for "insurance purposes".

The sample required for total IgG estimation is filtered and fully processed. This sample is stable at -70°C for 3 months.

STANDARD MATERIAL

Protein reference standard SPS-01 is diluted in Diluent as detailed on page 4.

QUALITY CONTROL MATERIAL

A patient who is suspected of having raised inflammatory parameters is asked to give consent to have his sample tested for Hepatitis B and HIV infectivity. A large volume is stored at -70°C. If this sample is negative and the values are appropriate, the frozen material is thawed, filtered, processed and aliquoted into 500µl aliquots and frozen at -70°C. If the IgG level is inappropriately high then it can be diluted with "Klean-Prep".

Ideal values for the QC material are at the upper limit of the reference range.

(Refer to QC documentation in the Protocolfile for details of the current materials used)

ANALYSIS PROCEDURE

1. Coat plate with appropriate antigen
   Use Immulon 1 flat bottomed plates. Prepare a template of the sample positions.
   Dilute IgG coating antisera in carbonate/bicarbonate coating buffer pH 9.6 as detailed on page 4.
2. Using an 8 channel multichannel pipette, dispense 125 µl coating solution to each well of the Elisa plate .Cover plate with a plastic lid, place in a moist box and incubate at 4°C overnight.

3. Block Plate
   Either manually or using the Dynatech M-RW wash the plate x 3 and then add 250 µl Diluent. Leave for a minimum of 1 hour.

4. Prepare samples
   Dilute the standard material as detailed on page 3
   Allow lavage samples and QC material to reach room temperature.
   Dilute the samples and QC material 1/25 in Diluent (add 40µl sample or QC material to 960µl Diluent)

5. Add samples to the plate
   Dispel the blocking solution from the plate and blot dry on absorbant paper
   Using the 8 channel multipipette add 125µl of diluent to all wells except for B1,B2 and

A3-A12
To B1,B2 and C1,C2 add 125µl of the appropriately diluted standard. Mix the standard solution and the diluent in C1,C2 by drawing up and down the pippette tip 3 times. Then,transfer 125µl from C1,C2 to D1,D2 and so on to produce a series of doubling dilutions.
Similarly, after vortex mixing the diluted lavage samples, add 125µl to duplicate colums (eg.A3,A4 and B3,B4). Then by mixing the sample and diluent in B3, B4, produce doubling dilutions of the samples.

6. Cover plates with a plastic lid and incubate overnight at 4 °C in a covered moist box.

7. Conjugate
   Dilute the alkaline phosphatase conjugated antisera as detailed on page 4.
8. Wash the plates as above.
9. Add 125 µl of conjugate to each well using the 8 channel multipipette.
10. Cover the plate with a plastic lid and leave in the corridor incubator at 24°C for 3 hours.

11. Colour Development
    Prepare alkaline phosphatase substrate (15 mls per plate) 30 mins before it is required.
12. Wash the plates as above.
13. Add 125µl of prepared substrate to each well using an 8 channel mutipipette. .Leave the plates on the bench for 5mins as the colour starts to develop, then shake on the Wellmixx.
14. Monitor the colour development manually on the Microplate Reader and as the top standard nears its endpoint START the programme.
    Absorbance endpoint for IgG = 1.0
15. Programme for total IgG = 11. This programme will automatically subtract the blank absorbance values (wells A1,A2) from all wells.

CALCULATION OF RESULTS

Results can be calculated using a pocket calculator with statistical programmes, using the software programme on the Dynatech Microplate Reader or using the Minitab statistical package on the lab p.c.
The following method uses the first of these options.
Draw out a standard curve of absorbance readings against concentration of immunoglobulin at each dilution of the standard. Using the linear portion of the curve, calculate the linear regression line for these standards. Check the correlation coefficient.
Using the linear regression equation calculate the concentration of the QC and each sample at several dilutions

ACCEPTANCE OF RESULTS

The correlation coefficient for the standard curve must be greater than 0.985.
Sample results from plates on which the QC value falls within 95% of the current QC mean value can be accepted for reporting.
Samples must have 2 or more points parallel to the standard curve and a mean of the concentrations at these parallel points is taken as the result.
Samples with absorbances above those of the standard curve are repeated at a starting dilution of 1/250 (add 4 µl lavage to 996µl Diluent)

REPORT FORMAT

Results for lavage total IgG are entered into the REPORTS database, reported to the nearest whole number. Samples with very high results that have required dilution are

reported to the nearest 10. The results are reported as one of the "inflammatory bowel assessement / blood loss" parameters and printed using the mailmerge programme.

Lavage total IgG reference range 0-10μg/ml

CLINICAL SIGNIFICANCE OF RESULTS

Total IgG is the most sensitive parameter to inflammation in the gut. The relevent comment is
"Results indicate active inflammation".

METHOD VALIDATION

Performance characteristics
Lavage total IgG : Between batch CV =12.3% at 19.8μg/mli